

## Public enterprise and private sector

*All governments live with nationalized industries, some more willingly than others.*

*Ideology apart, all of them need better guidelines.*

LIKE the poor, and often for the same reasons, nationalized industries are always with us. Even where the prevailing ideology is dead set against the notion that government should shoulder tasks that might be put out instead to private persons, as in the United States, nobody would seriously suggest that national defence should be put out to tender. Warfare being what it has become, even the largest private organizations would probably find the defence of all but the smallest realms beyond them, while those occasions when one state provides for another's defence are usually in the long run regarded resentfully as unwarrantable intrusion by those defended. Similarly, most governments take it for granted that the civil service should be nationalized — and should therefore look more carefully at the Swedish tradition of putting out all but the most sensitive policy-making tasks to more or less autonomous agencies.

These categories apart, however, countries vary in their attitudes towards nationalization in such a way as to cover the whole of the accessible spectrum. In the United States, the principle that the government should never take on tasks that the private sector might attempt is widely preached and zealously practised, as when the National Aeronautics and Space Administration is persuaded (unsuccessfully so far) to sell off its meteorological satellites and the services they provide. Elsewhere, as in India, public enterprise is not merely a necessary but also a virtuous way of doing business, on the grounds that the government deserves a stake in enterprises it has sponsored (and often because it knows that enterprises such as airlines and broadcasting systems would not exist if it were not willing to pay the cost with taxpayers' money). And then, of course, there are the socialist states of Eastern Europe, where the ownership of productive resources (from factories to farmland) on behalf of the community at large is a properly consistent part of a government's responsibility. What is to be made of these huge disparities of practice?

### Inheritance

The past few years have been enlightening. For different reasons, governments seem to be passing through a phase of discontent with what they have inherited from their predecessors. In the Soviet Union, for example, there seems to be yet another attempt deliberately to experiment with schemes that will allow the managers of factories more freedom to run their plants efficiently, and not by some rule-book provided by a central ministry, which does not of itself compromise community ownership. France, on the other hand, has recently lunged in the opposite direction and has nationalized various banks and industrial companies on the grounds that its political supporters, having chosen freely at the polls, require no less. If past experience in Britain is any guide, the next government of a different complexion will just as hastily embark on the opposite course. Meanwhile, Britain seems embarked on a phase of denationalization for reasons that are partly ideological, in the mould of the United States, but severely practical as well: the British Government is hoping more successfully to balance its accounts by selling off to the private sector as many saleable public enterprises as it can. Again, as in France, the policy is strictly legitimate, although many people may disagree with it. What seems to have been overlooked, by all the governments now preoccupied with the proper place for public enterprise, is that title to ownership may make little dif-

ference to practical performance — and that it is easy for over-hasty reorganization to make things worse for everybody, those who work in an industry and those who use its services included.

Britain is where the action is just now — only last week, the rumour took hold that the government, having decided in its last tenure of office to sell off the retail shops operated by the British Gas Corporation on the grounds that they could just as well be operated by private persons, has now taken the decision to sell a major shareholding in the corporation as a whole. There is one important objection of principle to this and other schemes: good housekeeping suggests that the sale of capital assets to pay current bills (such as unemployment pay) is poor financial practice. A few case histories from elsewhere should show the British Government that there are pitfalls of a more practical kind, some of which it may have overlooked.

### Default

There is, for example, the case of the Washington Public Power Supply System, the electricity supplying electric power in the northwestern United States which embarked on an ambitious nuclear power programme in the 1960s, which decided last year that it would not be feasible to complete two of five reactors, and which was declared in default on its debt a week ago. Like other utilities in the United States, the company has traded its virtual monopoly of the right to sell electricity in the state of Washington for a restriction of the right to charge whatever prices come into its head by a state regulatory commission, which takes account of such things as the likely return on capital. But regulation of prices does not, of course, prevent a utility from making mistakes, whence the present plight. In a strictly commercial world, the consequence would be that the company would now go out of business, and its assets would be sold off to the highest bidder. No doubt among the pieces to be picked up would be a few operating power plants, which might be operated by new owners if the regulatory authorities would agree or otherwise sold for scrap, but in the meantime residents of the state of Washington would be without electric power, which is politically unthinkable. So one way or another, the state and federal government will arrange that the completed power stations will keep on working, some of those who have invested in the system will lose their money (the courts will eventually decide which) and the demonstration thus provided that even electricity utilities can get into financial trouble will increase the cost of financing power stations elsewhere in the United States (which is another way of saying that other electricity consumers will eventually pay the cost of what has happened). The moral is that there are some kinds of industries of which governments cannot permanently wash their hands.

The case of AT&T's long travail in the courts and in Congress is more relevant for the British Government. After more than a decade of fending off anti-trust suits, it has been agreed that competitiveness will be best served if AT&T is broken up into a number of regional telephone companies and a central company free to compete with other providers of modern telecommunications services. One snag, now, is that some of the deregulators have begun to have cold feet, not just because an industrial giant has been broken into parts but also because it may be many years before would-be competitors can look even the parts of AT&T squarely in the eye.



None of this argues against denationalization of the kind being canvassed in Britain but, instead, for old-fashioned caution. Selling off the British telecommunications (planned for the past two years) is technically feasible, but the British Government's proposals are too much like a transfer of a public monopoly right directly to the private sector. On the grounds of the equitable treatment of would-be retailers, the proposal to strip the British Gas Corporation of its retail trade makes sense, while it would similarly be proper to make the corporation confine itself to its main business and not to go exploring for natural gas in the North Sea, which is a needless risk of public money. (An onshore crude oil field in the south of England is to be sold.) The plain truth is, however, that whatever happens to the corporation or to the other nationalized industries canvassed as saleable, the government will have no choice but to step in if, in the last resort, privately-owned successors fail to do what is expected of them. And in the meantime, there will have to be devices for protecting consumers against the abuse of monopoly rights, which means that regulatory bodies will have a hand in fixing prices and that price competition can never be as naked as economics textbooks wish.

The moral, for the British Government and for other governments of different persuasions, is that the titular ownership of public functions, utilities, some transport systems and also social services such as health and education, is less important than the means other than price by which their performance is regulated. And since some nationalized industries will remain even when the most saleable assets have been disposed of, governments such as the British should give more thought than is their inclination to devising a framework in which public tasks can be carried out efficiently, in which innovation is encouraged (and not discouraged) and in which there is an equitable balance between the interests of consumers and those who finance these services. The British Gas Corporation (which made a profit of more than £600 million last year) may be saleable, but who would think of selling British Rail (which cost taxpayers almost as much)? □

## Skin of all our teeth

*US Congress increased funds for the International Monetary Fund without sensing the dangers.*

THE principle that people forced by circumstances to bow to the inevitable prudently do so with a good grace seems not to apply to the US Congress. A few hours before the beginning of the summer vacation last week, the House of Representatives grudgingly agreed that the United States should contribute a further \$7,000 million to the International Monetary Fund (IMF), the bank from which only governments can borrow. Most of those participating last Wednesday in the last-minute decision will be free to spend the next few weeks making tentative alliances against the 1984 presidential election oblivious of the near-disaster that has been narrowly averted (by 217 votes to 211).

Parochially occupied as they are, US congressmen are not alone in asking why IMF's capacity to lend to bankrupt governments should matter. To the extent that the prospect of a national bankruptcy is a sign that the government at risk has mismanaged its affairs, people elsewhere (but especially elected politicians) are tempted smugly to recite some version of a Victorian homily on the virtues of thrift and wash their hands of an unseemly business. Why all this trouble to save others from the their own folly?

The essence of any answer is the economic interdependence of the world in which even congressmen live. It is too often forgotten that before 1973, the volume of international trade was the most rapidly growing economic indicator, and that many poor countries were growing more quickly than, say, the old economies of Western Europe. The increased price of oil made some of them rich overnight, and the more recent slump in the real price of oil has left them high and dry. But poor countries without oil have been adversely affected in two ways by factors outside their control — first, the extra cost of oil and, second, the slump in the price of and demand for primary commodities caused by the oil-provoked recession. Meanwhile, arguments persist about the

recession itself — has it ended or not? — and about more particular questions such as the influence of the US Government's huge financial deficits on international interest rates and thus on the capacity of the poor countries to pay their bills — and the interest on past loans. The US Administration is conscious of these interactions — witness last week's concerted but abortive effort to drive down the value of the dollar — but congressmen still seem to think they can act in isolation.

The immediate objective of the IMF exercise is to give the debtor nations time in which to pay their debts, and thus save the commercial banks which are their chief creditors from ruin. Even the extra funds now being recruited will give IMF the capacity to lend only \$100 billion, less than a quarter of the demands that might be made on it. The measure of which Congress has made such heavy weather will reduce the chance of a global collapse, but will not eliminate it altogether.

If the recipe should fail, the result would be quickly to snuff out the fitful signs of recovery from recession that the past few months have seen, make still more cruel the plight of the poor nations of the world and postpone for several years the hope that the diligent use of technology may once again give people a sense that circumstances are changing for the better. Even merely to decrease the chance of running into such a disaster is cheap at the price — especially because the US Government will not have to hand over £7,000 million in gold but merely has to promise to print the money if the need should arise.

Why is the House of Representatives so unwilling to accept these simple connections? The insularity of the most prosperous nation in the world is part of the explanation, as is the pursuit of political popularity by pandering to the short-term self-interest of voters while ignoring the frequent conflict between short-term and long-term interests (whence the protectionism now rife). But a more powerful influence on the present Congress has been the Reagan Administration itself, which came round only reluctantly to the notion that the fund's resources should be increased by about a half. Its more recent arguments in the opposite direction are necessarily unconvincing, but they have also been needlessly parochial. So it might have been predicted that the House of Representatives would wrap its concession on the major issue in a mass of restrictions of the commercial banks' freedom to lend outside the United States. The effect would be to cancel out the benefit of a larger IMF. Luckily, the US Senate has passed a more enlightened bill, but has yet to hammer out a compromise with its more electorally conscious legislative partner.

What will happen next is anybody's guess. The administration will be hamstrung until it gets its bill, which may not materialize until next year, in which case some promised matching contribution may also fail to materialize on 1 January 1984, the due date. Who can be confident that the fragile tracery of debt and rescheduled debt cobbled together in the past 18 months will last that long? That is a risk all will share.

All? What connection can there be between these arcane questions of how governments finance their continued existence, and the orderliness of that mechanism, and, say, the pursuit of research in academic laboratories of the application of technology to the real world? The scepticism implicit in that rhetorical question is easily dispelled by the evidence of the past decade or so. The temptation to seek a causal link between the inflation of the early 1970s and the stagnation of research budgets since that time, in countries such as the United States and many in Western Europe, should not be too vigorously resisted. Attempts since then by industrial nations to keep old-fashioned industries at work by restricting imports from elsewhere have prevented a more economical division of labour, impoverishing rich and poor alike. The result, in most countries, is that proven technologies — not just bright ideas — remain less than fully exploited for lack of resources, contributing to the general frustration and disappointment that marks the present. Financial mismanagement on a large scale is already directly the cause of many discontents, yet these are minor in comparison with what may lie ahead. Who, in these circumstances, will carry to the US Congress the simple truth that it no longer legislates for an island? □



## Fast reactors

# Britain ponders joining European collaboration

FAST breeder reactors are not dead in Britain, where the government seems to have turned down a plan to collaborate with the United States and to have turned to France instead. The plan seems to be to mount a collaborative development programme involving France, Britain, Belgium, Italy, the Netherlands and West Germany. But final agreement has not yet been reached.

The French fast breeder programme is now occupied with the construction of Superphénix, at 1,200 MWe the world's largest fast reactor. Superphénix should switch on in 1984, but the French and their partners have a problem with what to do next. Nobody, least of all the French, needs more electricity at present, so there is no market for the commercial demonstration fast reactors (CDFRs) that should succeed



CEA director, M. Rapin

Superphénix (except for a small military market for plutonium). Classing the reactors as part of a development programme, and sharing costs and expertise with the British, who have a long-standing fast research programme at Dounreay in northern Scotland, makes sense in France.

M. Michel Rapin, director of the technical research and industrial development wing of the Commissariat à l'Energie Atomique (CEA), is responsible for Superphénix and its successors. According to Rapin, "technical people" on both sides of the English Channel "now think we are very near an agreement". CEA had discussed cooperation with the United Kingdom Atomic Energy Authority (AEA) (which runs Dounreay) in 1979 and 1980 without success, but last year discussions began again after the matter had been brought up at a meeting between Prime Minister Margaret Thatcher and President François Mitterrand. This time the discussions went better. "Now it's a political matter", says Rapin.

In Britain, AEA admits it has recommended a foreign partner to the government — but coyly will not say which. The

recommendation follows a request last November by Mr Nigel Lawson, then Secretary of State for Energy, to the AEA chairman, Sir Peter Hirsch, to submit plans for a rationalization of the British fast breeder programme. There would be no series orders for fast breeders before the next century, Mr Lawson said, so the research programme should be trimmed. Mr Lawson asked Sir Peter to consult industry and to consider foreign collaboration.

Sir Peter sent his reply to the government early this year. It included alternative collaboration proposals with the United States and with Europe, one of them earmarked for approval. A government decision based on Sir Peter's recommendations was expected at the end of last session, but the general election, and the appointment of a new Secretary of State for Energy, has pushed the likely date of the announcement back to the autumn.

The outline European agreement — it would be between the five national partners in the Superphénix licensing company SERENA and Britain — falls into three main sections, says M. Rapin.

- First, collaboration on research and

development towards a CDFR. This would probably be Superphénix II, already under development, and likely to be sited next to Superphénix I or nearby at Marcoule on the Rhone.

- Second, commercial collaboration between British and continental nuclear constructors, in relation to CDFR and future fast reactors.

- Third, cooperation on the plutonium fuel cycle. (Here, Britain believes its Dounreay reprocessing system is considerably in advance of the French.)

Unlike previous attempts at agreement, where the French were said to be asking a £20-£25 million entry fee, this time the draft agreement mentions no money. Payment will be "in kind", says Rapin. Thus, for example, when Superphénix II is built, the British will be expected to construct part of it. Other CDFRs should go up in other countries, also paid for by cooperation, but these would come after Superphénix II, Rapin believes. They are not part of the agreement.

Thus arguments about the siting of a CDFR in Britain would appear to be very premature — although reports in a British Sunday newspaper that a British CDFR might not be built in Dounreay have angered Scots people in the region, who have learned to love the breeder as a source of jobs, rare enough in Scotland. Considering the local environmental objections that are likely to be raised anywhere else in the country, that might be argument enough to place it there.

Robert Walgate

## Arms control

# Remote seismic station on show

Washington

THE meeting of the International Union of Geodesy and Geophysics at Hamburg next week will be given a demonstration of a prototype of a seismic station whose development has been sponsored by the Defense Advanced Research Projects Agency (DARPA) of the US Department of Defense. The system, which depends on a custom-designed blend of hardware and software to extract digital information from analogue seismic signals, was demonstrated on 13 July in Geneva to a meeting of the working group on seismic detection of nuclear explosions, one of the technical committees operated by the United Nations Committee on Disarmament whose objective is to improve the remote monitoring of existing and (perhaps) future treaties on nuclear tests.

The seismic analysis system consists of a specially-designed microprocessor with a memory capacity of 1 megabyte (MB) together with disk storage of 86 MB and magnetic tape storage *ad lib*. Ordinarily, the system would be coupled to at least three local seismic detectors, but it is able to handle the output from seismographs sensitive at various frequencies.

The hardware has been developed by

Sun Systems of Mountain View, California, and the software by the company called S-Cubed of San Diego. The total cost of the system is said to be less than \$50,000, which it is hoped (by DARPA and its two contractors) will make purchase attractive both to governments seeking an independent capability for monitoring seismic signals and to seismologists with a more academic interest.

The long-term aim at Geneva is that national governments will use such systems for recording seismic data, and will agree to supply these by means of the World Meteorological Organization's communications network to two international centres, in Moscow and Washington. In return, they will be able to interrogate the centres for data from the global network on any chosen events.

Part of the interest of the new device is that it shows how the US Government has kept up its interest in seismic detection since the ending of the nuclear test-ban negotiations in 1980. To test the working of a global network, DARPA has recently established a Center for Seismic Studies near Washington, initially to use data gathered from seismic stations within the continental United States. □



## UK research councils

# Another inquiry under way

BRITISH support for civil science is destined for yet another shake-up. The British Government has commissioned a one-man inquiry into the organization of the five research councils, which between them spend more than £500 million a year on a blend of academic and applied research at British universities and research institutes.

The inquiry will be carried out by Sir Ronald Mason, for five years until the end of 1982 Chief Scientific Adviser at the Ministry of Defence, and who has now returned to the University of Sussex as professor of chemistry. Comparisons between the Mason study and that carried out in 1971 by Lord Rothschild are inevitable. Indeed, part of the objective of the new inquiry is to assess the working during the past decade of the Rothschild prescription, the most recent major upheaval in the organization of British civil science.

Rothschild's report (see *Nature* 234, 169; 1971) introduced two major innovations. The more contentious was the principle

appointment by two at a more junior level.

The scope of the Mason inquiry is as broad as Rothschild's, and its recommendations are also likely to affect the constitution of the research councils individually. The setting up of the Mason inquiry immediately on the heels of last week's publication of the major study of the relationship between universities and research councils (see *Nature* 4 August, p.383) suggests that the government, having been presented with some general principles, now wants action — and perhaps economies as well. But the new development will also be interpreted as a sign of the government's impatience with the results of that inquiry.

The timing of the inquiry is significant. Mason has been asked to report with recommendations by October, when there will still be time for the Advisory Board for the Research Councils to draw up recommendations for research council budgets during 1984-85 on assumptions which differ from those on which it is at present functioning, the continuation of the *status quo*.

Of the five research councils, the Natural Environment Research Council is probably in present circumstances the most vulnerable. Out of a budget of £86 million (in the year 1981-82), the council raised £82 million by means of research commissions from six departments of the British Government and from the European Community.

The council's spending on direct support for university research amounted to only £8 million, although the council also supports research ships and other facilities used by university researchers. Its most conspicuous single item of consumption is the Institute of Geological Sciences, which cost £28 million last financial year. Last week's report from the Morris committee suggested that the relationship between the institute and the universities should be "reviewed", which is polite language for suggesting that changes are necessary. Among many geologists, the existence of the institute is regarded as an impediment to the provision of adequate research support through other channels, such as the Science and Engineering Research Council.

As always on these occasions, the Agricultural Research Council can also look forward to an uncomfortable few weeks. A still unpublished report on the council precipitated the Rothschild inquiry more than a decade ago, the council was told last year that its real budget would be reduced from 1984-85 and it has a large proportion of its research (52 per cent) commissioned by a single source — the Ministry of Agriculture with the connivance of the agriculture parts of the administrations of Scotland and Ulster.

Absorption of the research council into

the ministry is certain to be one of the options considered in the Mason inquiry, and the same considerations are likely to be raised in respect of the Medical Research Council, whose National Institute for Medical Research was "fingered" in last week's report and which has not been forgiven for having persuaded the Department of Health to relinquish direct control over its "Rothschild money", largely by making a monkey of the chief scientist system.

The joker in the pack now to be reshuffled is the Department of Industry, whose formal connection with the research councils and universities is negligible, but which has been increasingly put forward in recent months as a source of opinion and financial resources. A more formal connection with the Science and Engineering Research Council (to which Rothschild's customer-contractor principle was not at the outset applied) could be on the cards.

Paradoxically, the research council most likely to escape untouched from the new review is that most complained of by the government in the past few years — the Social Science Research Council. Government criticism led to a formal review last year by Lord Rothschild which, among other things, recommended that the council could be left alone for the next three years. It seems improbable that Mason will defy this edict.

John Maddox



*Sir Ronald Mason — in Rothschild's footsteps.* that in respect of applied research, the titularly autonomous research councils should be regarded as research contractors working at the behest of ministries, themselves proxies for taxpayers, the ultimate "customers". As a result, parts of the budgets of the agricultural, medical and environment councils were formally transferred to the ministries most directly concerned. This stimulated a rare wave of public protest by research councils and members of the research establishment, provoked in part by the government's suppression of a rival prescription drawn up by Sir Frederick Dainton until the Rothschild review had been completed.

A second important feature of the Rothschild remedy was that government departments should appoint chief scientists competent to commission research from the councils and also to make judgements about the remainder of a ministry's research programme. The opinion that this part of the recipe has not functioned well is common. Most chief scientists have been short-term incumbents, while the agriculture ministry has even replaced one senior

## Krakatoa still active



THE volcano on the island of Krakatoa, still violently active today. In August 1883 the island was devastated and huge tidal waves were formed. This event, in which 36,000 people were killed, is commemorated in a centenary exhibition at the British Museum (Natural History) in London, running from 26 August to 25 October. □

## Satellite launchers

## Europe sees business ahead

EUROPE, and in particular France, is pinning great hopes on Ariane, the space launcher developed by the European Space Agency and now in the process of transfer to a private operator, Arianespace. French companies hold 59 per cent of Arianespace shares, followed a long way behind by Germany with 20 per cent. Britain holds 2.4 per cent, less than Italy (3.6 per cent) and Belgium (4.4 per cent).

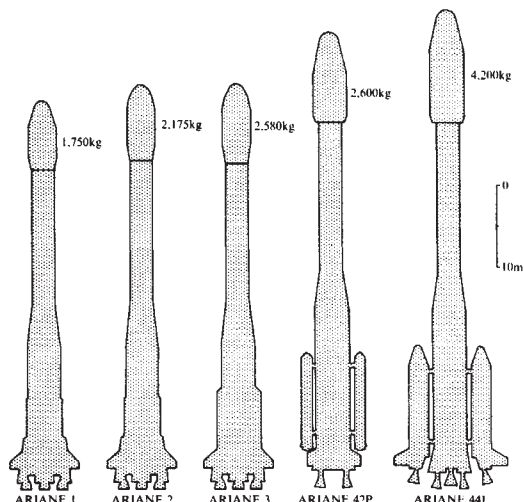
The company says it "will pave the way into the twenty-first century". Many doubt that Ariane can succeed in a battle for the business of launching commercial satellites, but in France especially there are those who point to the success of the French military aircraft industry in recent decades as a sign of what may be possible.

Out of five test launches, two have failed (the second and the fifth), but the causes are understood and have been rectified. After the second failure, the most disturbing, Arianespace was very frank with its potential customers about exactly what had gone wrong. Essentially, it was a matter of quality control in the manufacture of a fuel pump. With control now tightened up, Arianespace claims it lost no customers through loss of confidence: Western Union withdrew from one launch, but that was because the Ariane launch calendar was set back, and Western Union had its own tele-

communications customers to satisfy.

The key to Ariane's success now seems to lie in a continued series of successful launches (the next is due on 15 September), price relative to its competitors and accuracy. Accuracy is critical to a client because it means satellite lifetime. Ariane's last launch injected the European Communications Satellite ECS-1 into geostationary orbit so accurately that it needed only 2 litres of fuel to manoeuvre into its precision orbit instead of the expected 20 litres. The result is a fuel surplus that will give ECS-1 another year of life in orbit (on a planned seven years).

By contrast, the space shuttle's launch into geostationary orbit requires an inertial upper stage (IUS) which is both relatively inaccurate, claims Arianespace, and presently in trouble. IUS failed dramatically on 5 April this year, when the shuttle crew attempted to place a tracking and data relay satellite (TDRS-A) into geostationary orbit. An oil seal failed on IUS, throwing TDRS-A into a highly elliptical orbit. The long manoeuvre to get the satellite into the right position, using pitch control thrusters with only one pound of thrust each, was ultimately successful, but very costly in fuel. The IUS system is now under investigation, and the next shuttle/IUS launch has been put back to March 1984; it may be



## The Ariane series

ARIANE is actually a family of vehicles, based around the idea that communications and observation satellites (where all the business is) are likely to get bigger. Ariane 1 can carry 1,750 kg into geostationary transfer orbit. Ariane 2 and 3, for use from 1984 onwards, have a slightly bigger third stage and payload fairing, and two to four strap-on first stage solid boosters, raising the geostationary payload to 2,175 kg (Ariane 2) or 2,580 kg (Ariane 3). Ariane 4 is still under development at the French national space research centre, CNES, and will mark "a major step in the evolution of Ariane". Ariane 4 requires development of

new liquid fuel boosters, but these are being based around Ariane 1, 2 and 3 Viking engines. Ariane 4 payload mass to geostationary orbit should range from 2,600 kg (in version 42P, where the '2P' refers to two solid fuel boosters) to 4,200 kg for version 44L (four liquid fuel boosters).

For international comparison, apart from the shuttle the US Titan III-C can place 4,500 kg in geostationary orbit; the Soviet AZ Soyuz, 2,500 kg; the US Atlas Centaur D1 A, 2,225 kg; Delta 3920-PAM, 1,250 kg; and Japan's N2, 850 kg. Ariane 4 is scheduled for its first test launch in December 1985. □

## Engineering change

THE UK Science and Engineering Council (SERC) is worried that British industry is not taking enough notice of its engineering research — despite the fact that the council, under successive chairmen, has been edging more and more towards engineering. So it has commissioned the London-based Technical Change Centre (TCC) to investigate why industry ignores it, or as TCC puts it more sedately, "to study the way in which information is transferred between academic research institutions and industry".

TCC has begun work in disciplines in which SERC has already tried to improve industrial contact, by producing booklets which tell industrial scientists and engineers what SERC is doing. The areas are: noise and vibration, tribology and bearings, corrosion, grinding and dies and moulds. Has industry seen these booklets? Did they end up in the bin? TCC would like to know. Call Gil Collins or Carolyn Angell on 01-370 5770. Robert Walgate

delayed further if a design problem is discovered. Since the main pay-off in the space business is thought to be in launching communications satellites, this current problem for the shuttle is a boon for Ariane.

However, potential clients are more interested in having two alternative launch vehicles, both successful, one on either side of the Atlantic, than in backing "the winner", Arianespace believes. Even so, costs matter, and Arianespace looks askance at federal subsidies for the shuttle. Arianespace has none, the company claims.

According to Arianespace calculations, half an Ariane-3 (up to 1,195 kg payload) will cost \$25-30 million; and a shuttle flight costs \$250 million (excluding research and development), but could launch four satellites of the half-an-Ariane-3 size. This suggests that the true costs of the shuttle are twice those of Ariane. But the shuttle is asking only \$8-18 million during a "promotional phase", Arianespace claims. After 1986, the shuttle loses its subsidy, but launch costs should also be falling to around \$180 million. This should be the future purely commercial cost of shuttle launches, according to Arianespace. Ariane 4 costs are "unknown" at present. But clients are choosing both systems, Arianespace claims.

The only flies in this transatlantic balm are the American commercial company Marion Marietta, which hopes to undercut both the shuttle and Ariane using NASA Titan rockets, the Soviet offer to launch at "\$1 under" any Western price and Japan's efforts to miniaturize satellite technology so that smaller launchers (like Japan's N2) can be used. None, however, appears for the moment to be serious competition.

Robert Walgate



## AIDS research

# Big enough spending?

*Washington*

THE federal response to acquired immune deficiency syndrome (AIDS), the mysterious and uniformly fatal disease whose victims are almost exclusively homosexuals and intravenous drug users, has been a predictable target for charges of government discrimination. Would the government be doing more, critics ask, if the victims were Scandinavians or tennis players?

Despite the recent provision of \$12 million in emergency funds for the federal epidemiology and research effort, that question will not go away, and last week a House of Representatives subcommittee heard city health officials, homosexual rights activists, AIDS researchers and AIDS victims say that the government could be doing more, could be doing it faster and could be more candid in admitting that it needs more money to back up its declaration of AIDS as the "number one" public health priority.

The criticism of the federal role comes at a time when the number of new AIDS cases continues to accelerate. The Centers for Disease Control (CDC) reported late last week that an average of 53 new cases were reported each week in July, twice the rate recorded in January and nearly five times the rate recorded a year ago. New cases reported in the past six weeks account for 17 per cent of the 1,972 reported since 1981.

Nearly 90 per cent of the patients continue to be members of two high risk groups, male homosexuals and intravenous drug users; the remainder are haemophiliacs and others who have received blood transfusions, recent Haitian immigrants to the United States (although

there are increasing suggestions that many, if not all, are members of one of the high risk groups), and a few who do not appear to have any of the identified risk factors.

AIDS researchers seem generally to agree that research support from the federal government has now reached adequate levels. But the lingering question remains why it took so long for that support to become available and why, until a few months ago, federal health officials continued to insist that existing funds were adequate and that everything that could be done was being done.

Dr Marcus Conant, co-director of the Kaposi Sarcoma Clinic at the University of California, San Francisco, accused the Public Health Service (the federal agency that includes CDC, the National Institutes of Health and other health agencies) of playing a numbers game to conceal the inadequacy of support for AIDS research and monitoring. Earlier this year, Assistant Secretary for Health Edward Brandt, in announcing that AIDS was number one priority, asserted that \$14.5 million would be spent this year on the problem.

But Conant said this figure reflected a "double accounting" that included projects on immune function and cancer that were under way before AIDS was even identified and funds for which were appropriated as long as four years ago. Brandt, while not specifically denying the point, adds that if all basic research were included that could lead to treatment for AIDS, the figure would be \$166 million.

But both inside and outside the government there is considerable confusion over just how much new money has been made

available for new work specifically on AIDS.

Brandt stands by the assertion that enough money is being spent, and suggests that those who are "throwing numbers around" on how much is needed should back up their claims with specific advice on how the money should be spent. But under questioning by subcommittee chairman Ted Weiss (Democrat, New York), it became clear that Brandt's position on the adequacy of support has been a constant refrain, even during the time that Brandt himself was seeking to divert \$12 million from other areas to AIDS.

In April this year, Brandt and other Public Health Service (PHS) officials were telling a House appropriations subcommittee that no new funds were needed; just a month later, they were asking the same committee for permission to reprogramme the \$12 million. A memorandum from the director of CDC to Brandt itemized \$2.25 million in immediate needs, including \$1.5 million to restore funds previously diverted from other areas. Congress decided to go one better by appropriating the \$12 million as an immediate supplemental budget for fiscal years 1983 and 1984. "Federal officials who say that enough money is being spent on AIDS are simply mouthing some required political line", Conant said.

Another target has been what critics see as foot-dragging by the National Institutes of Health (NIH) in issuing research grants on AIDS. The first announcement of new grants specifically for AIDS research was published in August 1982, a year after the first cases were reported, and grants were awarded in April and May this year. Proposals in response to a second announcement were due this month and will be awarded shortly.

PHS officials say that the usual time for developing a request for proposals, reviewing applications and awarding grants is 18 months; the first AIDS awards were made 14 months after the go-ahead in January 1982 from NIH's scientific advisory council. So far, 14 grants have been made, or about 30 per cent of the approved applications — roughly the going rate for NIH as a whole.

The inertia of the NIH bureaucracy seems more to blame than any special indifference to AIDS, and Brandt has offered assurances that future research funding will be expedited. But the sudden influx of the new funds may add another bureaucratic tie-up; some AIDS researchers say there is now an undirected scramble by the various NIH institutions to get into the act, each supporting research and calling meetings on a particular aspect of AIDS that falls within their purview. Stanley Matek of the American Public Health Association urged Brandt to call a conference of experts within and without the government to establish a master research plan and avoid "shooting in the dark with scientific scatter guns".

**Stephen Budiansky**

## Confidential matters

"PEOPLE aren't going to answer questions about illegal acts truthfully to a federal official", is how one AIDS patient sums up a problem unique to this disease which threatens to undermine vital epidemiology research. "There is a need to reassure communities who have no reason to trust the government."

Homosexual rights groups say that before AIDS patients can be expected to reveal details of their sexual case histories and admit to illegal acts such as prostitution or drug use, the Centers for Disease Control (CDC) will have to do more than say "trust us". Incidents such as CDC's delivery of a list of names of AIDS patients to the New York Blood Center — perhaps by accident — have hardly allayed fears.

In response to this concern, and aware of the damage that underreporting or omission of important details from case histories could do to epidemiologic investigations, CDC has proposed a system whereby case reports from state and local

health departments would be identified only by number. Several local health departments, including New York City, have already adopted such a policy and are refusing to provide names to CDC.

CDC's efforts to reassure AIDS patients that their files will be kept secret may have bordered on the hypocritical, however, when it refused to supply information to a House of Representatives subcommittee investigating the federal response to AIDS (see main story). Representative Ted Weiss accused the Department of Health and Human Services of "stonewalling, from Secretary [of HHS Margaret] Heckler on down" when it denied subcommittee investigators access to CDC files. Calling the CDC's newfound concern for patient confidentiality "the height of cynicism", Weiss said that "we are not only uninterested in seeing names ourselves, we question whether CDC should have that information" in the first place. Last week, CDC was still refusing access to congressional investigators.

**Stephen Budiansky**



## Asbestos

## Conflicts ahead on UK rules

PROPOSALS for new safety controls on asbestos are high on the agenda for the meeting of the British Health and Safety Commission on 23 August. Asbestos has loomed large in the public consciousness for some time now, and seems likely to stay that way.

The most awkward document before the commission is a report from a working group under the chairmanship of Mr Steven Grant, senior director of the Scottish Health and Safety Executive. When the commission decided a year ago to tighten its control limits for exposure to asbestos dust, Grant was asked to identify and recommend new measures for control that were not considered at the last major review. His unpublished report, a copy of which was obtained by TV-am, argues that guidance given by the commission is inconsistent with the 1969 Asbestos Regulations.

The commission's guidance note for asbestos users lists "control limits" for the three main fibre types; if airborne fibre concentrations exceed the limits, protective equipment must be used. The 1969 regulations, however, seem to require the use of protective equipment wherever there is dust containing asbestos "to such an extent as is liable to cause danger to health". Grant says that because many factories now have exposure levels below the control limit, the "carrot is now behind the donkey": despite the lack of evidence for a safe exposure level, factory inspectors are reluctant to take action if control limits are not being exceeded.

He proposes an "action limit" for protective measures that would depend only on medical evidence of health dangers and feasibility of measurement. Grant's report also highlights apparently serious deficiencies in the way protective clothing and equipment are used in practice.

After Grant's report was submitted to the commission, there was an acrimonious correspondence between Grant and Mr William Simpson, the commission's chairman. Simpson wrote that the report could be seen as undermining the commission's advice, that the committee had exceeded its brief and that the report should be looked at again. Grant threatened to resign and the commission now says his report will be published unchanged after the meeting on 23 August. One thing the commission will want to know is whether the report represents the unanimous view of the working group; at least one member, Mr Albert Blyghton of the Transport and General Workers Union, is thought to have complained that there was insufficient time for its consideration.

A second report to be considered by the commission is an update of the evidence on health effects of asbestos prepared by Professor Donald Acheson and Dr Martin

Gardner of the Medical Research Council's Environmental Epidemiology Unit at Southampton. The authors have complained publicly that a press release they had agreed with the Health and Safety Executive was not published with the report. The executive says the document was held up because the commission had been unable to consider Acheson and Gardner's report fully before publication.

The report does not come to any startling new conclusions on medical effects, although the case that amosite (brown asbestos) is more dangerous than chrysotile (white asbestos) is strengthened and the linear relation between exposure to chrysotile and lung cancer mortality is supported. Though chrysotile is now almost the only type of asbestos used in UK manufacturing, Acheson and Gardner recommend a formal ban on the manufacture and importation of products made of amosite or crocidolite (blue asbestos) and call for further improvements in control to be made as engineering advances make them possible. Use of all types of asbestos should be reduced as substitutes become available. A separate full review is being prepared by Sir Richard Doll, warden of Green College, Oxford, although this will not be available until

towards the end of this year.

As if the two working group reports were not enough, the commission will also have to consider how enforcement will be affected by two new EEC directives finally passed in June but subject to a parliamentary reserve. Parts of these — such as the formal ban on crocidolite — will merely formalize changes that have already been made in practice. In other areas there will have to be some reorganization, as, for example, in the requirement for a formal notification and medical surveillance system.

Last but not least on the agenda, the commission will be looking at proposals for new legislation on the licensing of asbestos stripping operations. Work was recently halted twice during stripping of a disused power station at Fulham, London, after airborne fibres were detected. In response to public concern over Fulham, the Central Electricity Generating Board has now decided that it will in future itself take responsibility for stripping operations rather than sell power stations complete with asbestos insulation *in situ*. The Health and Safety Executive was much relieved that it will in future have to deal with only one authority to monitor the work: some 40 power stations are expected to be decommissioned over the next few years. The board's decision will cost electricity consumers about £1 million per power station.

**Tim Beardsley**

## Plant biotechnology

## Rockefeller seeks new success

THE Rockefeller Foundation in New York is to re-enter the field of agricultural research, but this time by means of a programme of plant molecular biology aimed at "putting the technology into the seed". As part of the programme the foundation has just made a grant of £230,000 to two scientists at the Plant Breeding Institute, near Cambridge, England. The grant will enable Dr David Baulcombe and Dr Michael Bevan to develop methods of introducing viral disease resistance genes into crop plants.

The Rockefeller Foundation's Agricultural Science Program has (under a variety of names) been sponsoring research aimed at improving food production in developing countries since the 1940s, when it established the first of the International Agricultural Research Institutes in Mexico. The institutes introduced improved varieties of wheat and rice that are now widely cultivated. Since the Consultative Group on International Agricultural Research took over the running of the institutes in 1971, the foundation has concentrated more on supporting specific research projects. Roughly one third of the Agricultural Science Program's \$7-8 million annual spending is now on plant genetic engineering.

The immediate aim of the research at

Cambridge is to investigate how cross-protection may be used to protect plants against tobacco rattle virus and cucumber mosaic virus, both of which cause diseases of agronomic importance. Characterized and sequenced genes from these viruses will be attached to plant promoter sequences and transferred into host plants using as a vector the transforming bacterium *Agrobacterium tumefaciens*. The researchers have already obtained a complementary DNA clone of the satellite cucumber mosaic virus. Tobacco plants will be used as hosts initially, to be followed by tomato and potato plants; these will be tested for resistance in collaboration with Dr B.D. Harrison, at the Scottish Crop Research Institute. If the idea proves workable, one of its applications may be in preventing diseases of potato crops.

As Rockefeller is a charitable foundation, one condition of its support is that research results are published and available to all interested parties. There was some concern during negotiations that the "first rights" agreement between the newly-formed Agricultural Genetics Company (see *Nature* 28 July, p.296) and the British Agricultural Research Council might prove to be an obstacle, but the foundation is now satisfied that its conditions will be met.

**Tim Beardsley**

## European Community

# Research programmes trimmed

## Brussels

ONE of the European Community's summer rituals is a decision by its finance ministers to slash planned spending on scientific research projects. The executive Commission always complies with little protest, for it knows that one of its own winter rituals is to reverse the decision, bringing research spending back to the level originally planned.

But this year, things may be different. Runaway farm spending and a rebate of 750 million ECU (1 ECU = £0.57) on Britain's 1983 budget contribution threaten to empty the Community's coffers before the end of the year.

Everybody agrees that something has to be done to relieve the Community's financial hardship, just as there is a consensus over the need to boost Community research spending. European leaders solemnly stated at their summit meeting in Stuttgart that "lasting solutions" were to be found at their next meeting, in December in Athens. The Athens summit will also deal with the Commission's proposal to increase the contributions of member states to the Community budget to allow for the development of European social, regional and scientific policies.

But it seems unlikely that the hoped-for extra money will be made available, and two of the principal victims will be the ambitious five-year framework programme for research and development of Vicomte Etienne Davignon, Commissioner for Research and Industry, and the ten-year, 1,500 million ECU strategic programme for research in information technologies (Esprit). Officials expect little from the Athens summit. "It's too short notice. At the most, they will agree on cutting farm spending, but national contributions won't be increased before 1985 or 1986 at the earliest", said one Dutch diplomat. "And as long as there is no fresh money, there will be no new policies — European Community laws state that farm spending is compulsory while spending on, say, research is not."

Davignon is unlikely to accept this point of view. First of all, he can count on the European Parliament to support more Community research. And so far, his battle for a stronger European research and development policy has not been unsuccessful. European research ministers have endorsed the outline of his five-year research and development framework programme and the need to increase Community spending substantially over the next few years. He has been congratulated on his efforts to attract European industry into Community-managed research schemes such as Esprit and has been urged to devise similar plans for biotechnology and telecommunications. "And these political statements will have to be

honoured financially", says Jean-Claude Brouwers, Davignon's principal aide for budgetary and scientific matters.

But Brouwers may be over-optimistic. At their marathon meeting on 22 July, finance ministers had little hesitation in cutting back on previously agreed support for next year. "This is very serious", says Rainer Gerold, Commission director for research budget matters. "Take our programme for radioprotection. The council reduces allowances by 4 million ECU to 7 million ECU — admittedly a small sum compared with total spending cuts of more than 90 million out of the requested 540 million ECU — but the loss cannot be made good in 1985 because the programme runs out at the end of next year. This means that we lack the financial means properly to evaluate the research results, and that the money we have invested previously has, at least partly, gone to waste. And this is only one example."

The Council of Ministers has been equally hard-hearted in allowing for new posts. In all, three new posts out of the 66 proposed have been agreed. "Another striking example of shortsightedness", says Brouwers. "We get the money to launch a programme, but we cannot hire the experts that have to run the show. Take Esprit. We expect the programme to employ more than 2,000 highly-qualified researchers in industry. But our application for 91 officials to run the programme, 25 next year, is flatly turned down."

In all, finance ministers reduced the Commission's demands for existing programmes by 47.3 million ECU to 239.6 million ECU, with sharp cuts in research on raw materials (from 17 million to 12 million ECU), fusion research (from 94.4 to 80 million ECU) and operating costs for the Joint European Torus (JET) at Culham in the United Kingdom (down 5 million ECU to 95 million ECU). New programmes have also suffered severe cutbacks, totalling approximately 32 million ECU out of the 111 million ECU requested, although Esprit seems to have been spared with allowances of up to 45 million ECU, or 3 million ECU less than was asked for and largely explained by the denial of posts for officials. The Joint Research Centre has to contribute its share in savings with cutbacks totalling 26.7 million ECU from a requested 192 million ECU for 1984.

The next negotiating round between Davignon and the finance ministers will take place after the summer. Before that, officials say, the Commission will try to convince the European Parliament, which has extensive budgetary powers, to restore the financial proposals to their original shape. But officials and diplomats agree that there is little ground for optimism about the final outcome of the negotiations in December.

Geert Linnebank

## Albanian science

# Flickers of interest again

ALBANIA is planning almost to double its scientific and technological "information base" by the creation of four new scientific centres under the Academy of Sciences. This decision, announced last week in a bland dispatch from the official news-agency ATA, appears to be related to the secret debate believed to be taking place among government and party officials about how far Albania can pursue party-leader Enver Hoxha's policy of economic self-sufficiency and at the same time bring its technology up to date.

This debate is believed by Albania-watchers to have resulted, in autumn 1981, in the "suicide" and subsequent denigration of Mehmed Shehu, for many years Mr Hoxha's closest lieutenant. During the past eighteen months, there have also been signs that the Albanian Academy of Sciences and Tirana University wish to expand scientific and academic links abroad, particularly in postgraduate training.

At present, Albanian scientists and technologists can gain access to Western research — and, for that matter, Eastern-bloc research — mainly through documentation centres at home. These include a Centre of Scientific and Technical Information and Documentation and the four existing centres operating under the academy which cover geology/mining, agriculture, oil and mechanics.

The four new centres announced by ATA will deal with energy, metallurgy, ore processing and oil technology. It is not explained how the competence of the existing "oil" and protected "oil technology" centres will be divided, but between them the eight centres will cover all the main Albanian primary production sectors.

The task of the centres, according to ATA, is the "gathering and systematization" of all domestic and foreign "studies, projects, articles and various data" in the relevant fields. Because of the small research base of Albania (the country has produced in all only 30,000 graduates during the 25 years of existence of Tirana University), it is clear that most of the information must come from foreign sources. Just how far the Albanians can utilize even middle technology without closer contacts abroad is somewhat doubtful.

The few hand-picked foreign visitors who have been allowed into Albania in the past eighteen months report that human muscle-power is still largely the basis of agriculture and the occasional prestige engineering project (road, railway, hydroelectric plant), while the foreign imports of the 1960s (before the total clamp-down), such as modern greenhouses from the Netherlands, have been allowed to fall into disrepair for lack of maintenance know-how.

Vera Rich



## High technology stocks

## Glamour fades in computers

## Washington

THIS has been a bad month for high-tech stocks on Wall Street, with the manufacturers of products at the cheap end of the computer market leading the way down. Three developments seem to have made investors take fright, one of which is the persistent rumour, unconfirmed but not denied last week, that IBM, having captured 25 per cent of the US market for personal computers since the introduction of its new machine last year, is planning to introduce a microcomputer later in the year.

Uneasiness in the microcomputer field goes back, however, to the disastrous results for the second quarter of 1983 announced by Atari, the home computer subsidiary of Warner Communications Inc. In July, the company, which provided the bulk of Warner products during 1981 and 1982, announced a loss of \$310 million, dragging the parent company down with it. Atari, once the proud retailer of "Pacman", seems to have been slower than its competitors to devise new computer games for its machines.

A similar slump at Texas Instruments for the second quarter has given the financial markets more solid foundation for anxiety. The company acknowledges that it lost \$119 million on the operations of its small computer division, largely because sales of its 16-bit RAM machine called the 99 4A model did not match expectations in the first half of this year. At the same time, in common with other chip-manufacturing companies, Texas Instruments has found the market for chips much more competitive than previously. Adjusting to the now reduced volume of production of the small computer, and helping dealers to move their large stocks with the help of discounts, has caused the main company to write off \$50 million.

These two developments have persuaded the stock markets that while the home computer market remains buoyant in the sense that machines and the software for them are still selling, it will for some time be very difficult to tell which manufacturers are likely to capture substantial shares of the market. And the increasingly cut-throat competition suggests that few of them will make substantial profits until their number has been reduced by bankruptcy or merger.

Even so, some predominantly small-machine manufacturers appear to survive almost unscathed. Commodore International, for example, is thought to be about to announce results for the second quarter that will be better than in 1982 — but even so, the stock has fallen by more than a quarter to just under \$50. At the other end of the market, companies such as IBM have more than weathered the storm.

Two other developments affect interest in stocks of cheap computer manufacturers, one of which is the steady trickle of

unflattering news. Last Friday, for example, the *Wall Street Journal* reported that Osborne Computers is laying off more than 200 people in California and that Fortune Systems of California made a loss of \$3

## Biotechnology index

## First faltering

BIOTECHNOLOGY stocks broke their year-long climb last month, falling victim to Wall Street's edginess over higher interest rates and an across-the-board setback in the fortunes of high technology issues during the last week of July.

Although *Nature's* index of 16 leading biotechnology stocks showed a slight gain for the month of 5 points, it was only the jump of AB Fortia (now known as Pharmacia) by 20½ points that prevented the index from showing its first drop since it began last summer. Part of the explanation of that dramatic change is the company's decision to modify the treatment of financial reserves in its accounts so as to conform with US rather than Swedish practice (see *Nature* 5 May, p.5).

Further rises in interest rates predicted for the coming months could spell continued uncertainty for the biotechnology stocks. While the first products of the smaller companies are beginning to appear, much of the income of these ventures remains interest income on their not inconsiderable cash reserves. That situation never goes down well with investors, who would just as soon put idle cash in the money markets directly rather than through the purchase of stock.

Molecular Genetics, for example,

million on sales of \$13 million after making its first small quarterly profit in the first three months. But Wall Street interest in high-technology seems also to have been moderated by the discovery that old-fashioned industries such as the automobile manufacturers have been reporting profits much improved in comparison with the recent doldrums. □

reported its first profitable quarter last month, coinciding with the introduction of its first product, a monoclonal antibody vaccine to prevent scours in newborn calves. The product, sold now only in Canada, brought revenues of \$69,000 for the quarter. But the company reported the cost of goods sold to be \$81,000; and but for a considerable interest income of \$668,000 (up from one-tenth of that a year ago), the company's \$271,000 profit for the quarter (\$0.40 per share) would have been wiped out. Molecular Genetics also reported revenues of \$1,131,000 for sponsored research, up from \$290,000 in the same quarter last year.

Sponsored research is playing a large part in the fortunes of the smaller companies, many of which are cashing in on the eagerness of the traditional and much larger pharmaceutical companies to buy a piece of the action in biotechnology. Genex Corporation last month announced a deal with Yoshitomi Pharmaceutical Industries of Japan to conduct research and development on a genetically-engineered microbe for producing human interleukin-2, the humoral factor known to stimulate proliferation of T cells of the immune system and which is being studied as a possible therapeutic agent for acquired immune deficiency syndrome (AIDS) and may also prove useful in laboratory work on cancer therapy and immunodiagnosis.

Stephen Budiansky

## Nature index of biotechnology stocks

| 12-Month high | 12-Month low | Company                          | Close previous month | Close 27 May | Change |
|---------------|--------------|----------------------------------|----------------------|--------------|--------|
| 100½          | 45¼          | Pharmacia (A.B. Fortia) (Sweden) | 77                   | 97½          | +20½   |
| 23¼           | 15           | Biogen (Switzerland)             | 15¾                  | 16¼          | +½     |
| 6¼            | 3            | Bio-Logicals (Canada)            | 5½                   | 4¾           | -1⅛    |
| 16⅞           | 7¼           | Bio-Response (USA)               | 14                   | 13½          | -½     |
| 19            | 11⅞          | Cetus (USA)                      | 17⅞                  | 15¼          | -2⅞    |
| 15½           | 8⅞           | Collaborative Research (USA)     | 15⅞                  | 11½          | -4⅞    |
| 39⅞           | 15           | Damon (USA)                      | 32¼                  | 28¼          | -4     |
| 34¼           | 16⅞          | Enzo-Biochem (USA)               | 30½                  | 28½          | -2     |
| 18⅞           | 10⅞          | Flow General (USA)               | 15                   | 12¼          | -2¾    |
| 49⅞           | 26⅞          | Genentech (USA)                  | 41¼                  | 45¼          | +4     |
| 17¾           | 8¾           | Genetic Systems (USA)            | 16⅞                  | 12⅞          | -4¾    |
| 20½           | 12⅞          | Genex (USA)                      | 18½                  | 18½          | 0      |
| 31            | 21¼          | Hybritech (USA)                  | 27¼                  | 26½          | -¾     |
| 22¼           | 13¼          | Molecular Genetics (USA)         | 21½                  | 19           | -2½    |
| 23¼           | 15½          | Monoclonal Antibodies (USA)      | 18                   | 15½          | -2½    |
| 65⅞           | 42           | Novo Industri A/S (Denmark)      | 63¾                  | 57⅞          | -6⅞    |

Closing prices are for the last Friday of the month. For over-the-counter stocks, bid price is quoted; for stocks on the American and New York exchanges, the transaction price. The index stood at 238 on 29 July, compared with 233 a month earlier. Data from E.F. Hutton, Inc.

## Chromosome abnormalities

SIR — The News and Views article by R.G. Edwards (*Nature* 26 May, p.283) draws attention to the fact that the incidence of various types of chromosomal abnormalities in human pre-implantation stages of development has yet to be established, although the possibility exists that the incidence of such embryos could be very high. The article was related to the report by Angell *et al.* in the same issue (p.336) of the finding of both haploid and aneuploid conceptuses following human *in vitro* fertilization. The single 7-cell haploid embryo that was successfully karyotyped was classified as maternal in origin and 22X, -17. This observation clearly demonstrated that haploidy due to gynogenesis or more likely parthenogenesis, even when associated with autosomal nullisomy, is compatible with at least a limited degree of viability.

Conditions which might induce parthenogenetic activation, such as the general anaesthetic given to the mother, or the nitrous oxide used for inducing pneumoperitoneum during laparoscopy, were present. Indeed, various studies have clearly demonstrated that a wide range of anaesthetic agents are capable of inducing parthenogenetic activation in mammalian ova. Once activated by this means, development beyond implantation of presumptive haploid embryos has even been reported<sup>1</sup>. Exposure of mammalian eggs to a sperm suspension may also induce parthenogenetic rather than fertilized development<sup>2</sup>, the various enzymes released by sperm from the acrosome region providing the necessary activating stimulus. In the mouse, post-ovulatory ageing of the oocyte before exposure to a sperm suspension undoubtedly facilitates parthenogenetic activation, and this factor is equally applicable following exposure to a wide range of activating stimuli.

However, it has yet to be established whether human oocytes are equally or possibly more sensitive than rodent eggs to these activating stimuli, at relatively shorter time intervals after ovulation.

Possibly of greater interest, however, was the high incidence of aneuploidy reported by Angell *et al.* While it is possible that a significant proportion of aneuploid oocytes may be ovulated in man, this would clearly be at variance with the stimulation observed in other mammalian species, where the incidence following both *in vivo* and *in vitro* fertilization is extremely low. This suggests that the methodology associated with the collection and fertilization of the material in their study might play a significant part in elevating the true incidence of meiotic non-disjunction.

The exposure of recently fertilized mouse eggs to ethanol while they were completing the second meiotic division induces a relatively high incidence of non-

disjunction in the oocyte-derived but not the sperm-derived chromosomes<sup>3</sup>. A similar effect is also observed when mice are anaesthetized close to the time of the HCG injection during induced ovulation<sup>4</sup>.

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1. Kaufman, M.H. *J. Embryol. exp. Morph.* 33, 941-946 (1975).
2. Kaufman, M.H. *Nature* 242, 475-476 (1973).
3. Kaufman, M.H. *Nature* 302, 258-260 (1983).
4. Kaufman, M.H. *Dev. Mammals* 2, 137-163 (1977).

## ARC's role

SIR — Your leading article on the Agricultural Genetics Company (AGC) (*Nature* 28 July, p.294) calls for clarification of my council's position. You are correct to emphasize the need for a strong flow of innovation between publicly-funded laboratories and British industry. This is what led the Agricultural Research Council (ARC) to enter into the agreement with AGC. By its nature, discovery in plant molecular biology needs further development and scale-up before it can be taken up by the agricultural supply industry. ARC scientists should concentrate on research and not be distracted by the need to study scale-up development nor to negotiate exploitation arrangements project by project. In ARC's judgement, AGC, with its specialist, technical and commercial staff and facilities, is just what is needed to bridge the technological gap between ARC laboratories and British industry. AGC's role in biotechnology can be likened to that of the Agricultural Development and Advisory Service in transferring ARC technology in more conventional fields of animal and crop husbandry. If, however, experience shows that the special relationship with AGC impedes the flow of innovation to the generality of industry, the problem would have to be addressed in the first review of the agreement, due in 1986.

Neither AGC nor my council intends the special relationship to exclude other companies continuing to collaborate with ARC institutes. As always, in their discussions with industry, ARC scientists must guard against the premature disclosure of potentially valuable commercial property and know-how. AGC recognizes that it cannot do everything in the field and so may be expected to have a general policy of welcoming ARC links with other companies and of exploring opportunities for joint ventures with those companies.

Nor is there any question that AGC will attempt to direct or influence publicly-funded research. That will remain the responsibility of my council and institute directors. It is likely, however, that AGC

will fund with ARC institutes, on a full cost basis, additional work relevant to its commercial objectives, as do other companies.

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(Secretary)

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## CO<sub>2</sub> euthanasia

SIR — I was startled to see, in a letter published in *Nature* last year, a casual mention of the fact that experimental rats had been killed by immersion in 100 per cent CO<sub>2</sub>. I have since discovered that this is a common method of killing animals, and is generally believed to be humane<sup>1</sup>.

Carbon dioxide is far from being an inert gas. Nunn, in his book *Applied Respiratory Physiology*<sup>2</sup>, devotes a chapter to its effects, noting that "few subjects in the field of physiology are as complicated" and listing five major effects on the brain, as well as major effects on the endocrine, respiratory and circulatory systems, the kidney and the electrolyte levels. Rebreathing from a closed system, so that the CO<sub>2</sub> levels steadily rise, causes the respiration to become so deep and fast as to be literally unbearable; the experiment is invariably terminated by the subject long before unconsciousness occurs. Although inhalation of 30 per cent CO<sub>2</sub> produces anaesthesia (through a narcotic effect and also by lowering the brain intracellular pH), this has never been popular as a human anaesthetic because of the frequency of convulsions. There appear to be no recorded experiments in which a human volunteer has inhaled 100 per cent CO<sub>2</sub> to the point of unconsciousness, and I have been unable to find a knowledgeable doctor willing to supervise that experiment on me. So there seem to be doubts, both physiological and humanitarian, over the use of 100 per cent CO<sub>2</sub> for killing animals.

In contrast, many human volunteers (including myself) have experienced simple oxygen deprivation, which causes no subjective suffering and few physiological consequences. Unconsciousness supervenes after a few minutes, unexpectedly and peacefully. Oxygen deprivation can be arranged by re-breathing via a filter that removes exhaled CO<sub>2</sub>, by inhalation of carbon monoxide, or by inhaling nitrogen. The last of these could be easily adapted for small animal work and would be safe for the operator. It might not be as quick as CO<sub>2</sub>, but it would certainly upset the animal's physiology less and might well be more humane.

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1. Baker, H.J., Lindsey, J.R. & Weisbroth, S.H. *The Laboratory Rat* 12, 24-26 (Academic, New York, 1980).
2. Nunn, J.F. *Applied Respiratory Physiology* 2nd edn, 361 (Butterworth, London, 1977).



# Nature is not all that malicious

*The proton is not decaying as fast as the simplest unified field theory — SU(5) — predicts. But this is no cause for despair: the search should be for something even simpler.*

It seems the whole world is delighted about the discovery of the intermediate vector bosons, the  $W$ s and the  $Z^0$ , which seem to fulfil all the requirements of the "electroweak" gauge theory developed by Sheldon Glashow, Abdus Salam and Steven Weinberg to unite electromagnetic and weak forces in one field of force. But there is a cloud on the horizon: the most obvious extension of the theory to include the remaining fundamental force apart from gravity, the strong nuclear force called "colour", which acts between quarks, is beginning to be "difficult to maintain". The words are Weinberg's, but others agree with him. The theory is minimal SU(5). (The symbols refer to a set of operations — a group — under which the "charges" of the theory are symmetrical.) "It's too early to draw the most discouraging conclusion", says Weinberg. But the experimental auguries are bad.

The immediate problem is an experiment which shows that quarks in protons (which contain three quarks) do not decay fast enough into positrons (anti-electrons). All unified theories that include colour inevitably draw a link between leptons — such as the electron and the neutrino, which do not respond to the colour force — and quarks, which do; and since quarks are heavier than leptons, the theories expect the former to decay into the latter. The rate of decay is predicted to be very slow, determined by the enormous energy, some  $10^{15}$  GeV, at which quarks and leptons should behave on an equal footing.

There are now four working experiments to detect nucleon (neutron or proton) decay, with others under construction, and there is some conflict among their results. All are deep beneath the earth, where they are shielded — more or less — from cosmic rays, whose most penetrating components are muons and neutrinos. The Tata-Osaka-Tokyo collaboration is running a 140-tonne detector at the Kolar Gold Mine, some 7,600 m of water-equivalent deep beneath south central India: they claim three contained decay events in the chamber, and three whose tracks pass through the chamber walls. The Frascati-Milan-Turin experiment 5,000 m (water-equivalent) under Mont Blanc, with a 150-tonne detector, has one event.

But the latest results come from the Morton salt mine, 1,600 m (water-equivalent) beneath Ohio. There the Irvine-Michigan-Brookhaven (IMB) collaboration, together with colleagues from Caltech, Hawaii,

Cleveland and University College London, has put 8,000 tonnes of pure water under observation by 2,048 photomultiplier tubes. Measurement is restricted to the inner 3,300 tonnes — but this is still 20 times the mass of the other experiments. The tubes and associated electronics and software are arranged to collect flashes of Cerenkov light that would arise if a proton in the water decayed. But at least in the first 130 days' running none of the 230,000 flashes collected each day was from a proton decay. The events, say the team in a paper in *Physical Review Letters* (51, 27; 1983), are mostly cosmic-ray muons passing through or interacting in the chamber of cosmic neutrino interactions. This has pushed the proton mean lifetime (in this mode) to something longer than  $10^{32}$  years at 90 per cent confidence, the collaboration claims. The SU(5) prediction was  $4.5 \times 10^{29}$  years, with uncertainty of  $\pm 1.7$  in the exponent.

This IMB result has cast doubt on the world's four other examples of nucleon decay in smaller detectors — even though they have much lower muon backgrounds (Kolar, 2 per day; Mont Blanc, 20 per day) — and physicists now appear to be taking the pessimistic view: they believe IMB, and dismiss the other results as flukes or misinterpretation. Moreover, although IMB has been looking predominantly at the pi-zero/positron decay mode predicted by SU(5), the experiment is also "sensitive to nearly every sensible decay mode" of the proton says Maurice Goldhaber, one of the team leaders. So an IMB search for decays into an alternative mode of K-zero/muon, for example, has put a limit on such decays at  $1.4 \times 10^{31}$  years, says Goldhaber. This begins to stretch another (more accommodating) unified theory known as "super symmetry", which attempts to link the description of matter with field. Supersymmetry predicts a dominance of K-decay modes.

Weinberg is naturally disappointed. "We had looked forward to happy years of exploring the phenomenology of proton decay. . . It would have been such fun to do all that." His view is that nucleon (proton or neutron) decay is likely to turn up some day, in the neighbourhood of  $10^{31}$  to  $10^{33}$  years, but this small range covers "the difference between instant gratification and years of subterranean labour". Assuming the latter holds, says Weinberg, "I think the only area where we're almost sure to see something

decisively important" now is in multi-hundred GeV collision experiments, to seek the mechanism which "breaks" the symmetry of the Salam-Weinberg model. This mechanism is usually assumed to be a set of "Higgs fields" a new form of matter which would fill the  $W$ s and  $Z^0$ .

Another thing to discover would be certain peculiar partners of the gauge particles and the fermions, called gauginos and sfermions, predicted by supersymmetric theories. Finding these would be even more exciting than proton decay, Weinberg believes, as supersymmetry is the most comprehensive of unified theories. However, "No-one so far has a model, nor a satisfactory picture of how supersymmetry is broken" says Weinberg. "If there were a candidate supersymmetry theory that didn't already disagree with experiment, that had naturalness and attractiveness, then I would say this is the most important thing, stop everything; but there isn't such a theory." Abdus Salam, as you might expect, is less pessimistic: he believes the content of the theories is relatively little explored, and that complicated effects within them might yield the world we know. He compares the problem with nuclear (as opposed to particle) physics. The phenomena may be an essentially complicated manifestation of a simple underlying structure. The vacuum may be like the nucleus, and it will take time to calculate its properties from supersymmetry (broken by supergravity, in Salam's view).

But Weinberg and others believe something new is needed. Weinberg and Philip Candelas are turning to geometry — theories that add extra, curled-over dimensions to space-time. "These calculations are extremely difficult mathematically, and we can deal only with spheres", says Weinberg, "but it may be that the real manifolds are more complicated".

However, whether Weinberg, Salam or some other is closer to the truth, no physicist should now forget Einstein's remark that God may be subtle, but not malicious. To make SU(5) fit Nature, the Higgs parameters (and there are 29 Higgs fields in the model) had to be extraordinarily finely tuned — certain arbitrary numbers had to cancel (but not quite!) to twenty-six decimal figures, without explanation for the cancellation. The theory surely deserved to go! Nature is *not* malicious: if it is, physicists might as well stop work. Simplicity has always been the answer, and will be yet. **Robert Walgate**

## Population genetics

# Tissue rejection: the price of sexual acceptance?

from J.S. Jones and Linda Partridge

MANY patterns of animal behaviour depend on an ability to identify and respond appropriately to close kin. These patterns are paralleled on the cellular level by the immune system's capacity to differentiate 'self' from 'not self' and to respond to inherited differences in cell-surface antigens. Remarkably enough, it now seems that the individual cues of identity present on the cell surface may lead to preferences in mating behaviour and to differences in mating success between animals having different degrees of genetic affinity. Sexual selection may therefore help to explain the extensive histocompatibility polymorphism found in natural populations.

Too close a genetic relationship between parents may lead to inbreeding depression<sup>1</sup>. In some animals, extensive dispersal of young makes consanguineous mating unlikely<sup>1,2</sup>. However, in others more specific behaviours reduce inbreeding. For example, young female chimpanzees associate with their brothers until puberty, when their behaviour changes and they avoid male relatives<sup>3</sup>. Mice also prefer to associate with unfamiliar mates<sup>4</sup>, and copulation frequency increases when a sexually sated male mouse is presented with a new and unrelated female; a phenomenon known, after the sexual habits of the American president, as the Coolidge effect<sup>5</sup>. Monkeys, mice, frogs and bees can distinguish kin from unrelated animals even when neither has previously been encountered<sup>6</sup>, suggesting that at least part of their discrimination behaviour has a genetic basis. This behaviour has evolutionary importance, as the offspring of *Drosophila* allowed a free choice of mate are more fit than those resulting from matings in which only a single partner is available<sup>7</sup>.

Mate choices which favour outbreeding must involve cues for the recognition of close kin. These have been most studied in insects. Honey bees produce inherited recognition odours which allow them to discriminate between half and full sisters even when they have been raised on the same comb<sup>6,8</sup>. In the sweat bee *Lasioglossus zephyrum*, males remember the odour of a particular female (or her sisters) for several hours, even when other females have been encountered in the interval<sup>8,9</sup>. Odour differences may also lead to mating preferences in *Drosophila* although there is disagreement as to whether females prefer an unfamiliar courtship scent and whether chemicals identified as pheromones in fly extracts are volatile enough to affect natural mating behaviour<sup>10-12</sup>.

In mammals, odours often act as individual sexual recognition cues<sup>13</sup>. For example, pregnant female mice and wild

horses may resorb their fetuses and return to sexual receptivity when presented with an unrelated male<sup>14,15</sup>. In mice this is known to involve scent as resorption occurs when male urine alone is used. The role of pheromones in human sexual behaviour and individual recognition is uncertain. However, we do differ in our individual odours in a manner which has a genetic component, as tracker dogs discriminate less easily between the scents of identical twins than between those of unrelated individuals<sup>16</sup>.

Scents are a manifestation of the uniqueness of the individual — cues that allow receptive animals to distinguish 'kin' from 'not kin'. There are similarities between this ability and that of the immune system to separate 'self' from 'not self'. In both cases, surface receptors of the appropriate chemosensory cells allow the animal to recognize and respond to minute quantities of a highly specific alien substance<sup>17</sup>. In both, perception of foreign substances depends on early experience: new-born mammals injected with an antigen are tolerant to it in later life, and young bees and mice presented with a foreign odour do not regard that odour as alien when exposed to it as adults<sup>18,19</sup>. Such 'phenotype matching' of an animal's own cues to that of a sensory template learned from close relatives in early life may be an important mechanism of kin recognition<sup>20</sup>. This could involve either genetically based scents, or cues learned from a common environment.

New evidence suggests that the analogies between the two sensory systems are even closer than at first appears, as mice of different histocompatibility type appear to produce distinctive odours that allow them to identify each other by smell. It is possible to produce inbred lines of mice which differ from each other almost exclusively in the genes coding for the major histocompatibility antigens. Such mice can be trained to choose the arm of a Y maze scented by urine from a mouse of the alternative histocompatibility type<sup>21</sup> and will subsequently recognize other mice of that antigenic type. Although environmental cues can influence mouse choice behaviour<sup>22</sup>, this is probably not the case in the histocompatibility-based choices as the animals used were F<sub>2</sub> segregants from the same litter who shared a common environment. Even mice whose antigenic differences involve only one part of the H-2 complex, an adjacent region of chromosome 17 or the *I* locus (15 map units from H-2) can distinguish each other<sup>23,24</sup>. Antigenic variation may therefore be a very effective mechanism for the recognition of individual mates.

Scent cues associated with cell-surface

polymorphism also affect mouse sexual behaviour. There is a strong odour-based bias by females against mating with males heterozygous for *I* alleles (which would have harmful effects in their offspring)<sup>24</sup>. Pregnant females are more likely to block implantation when presented with a foreign male whose histocompatibility type differs from that of their previous mate<sup>25</sup>. Moreover mice of different histocompatibility type sometimes prefer to associate with each other, and males prefer to mate with females whose cell-surface antigens differ from their own<sup>26</sup> (although the evidence here is less satisfactory as different inbred strains and not F<sub>2</sub> segregants were used). As close relatives are likely to share a high proportion of their cell-surface and odour cues such preferences will favour matings with unrelated animals and hence reduce inbreeding.

Sight is more important than smell in our choice of sexual partners. Human cell-surface polymorphism may nevertheless act to favour outcrossing because of its effects on fetal survival. About three-quarters of fertilized eggs fail to come to term<sup>27</sup>. Some of these losses may result from an interaction between the histocompatibility antigens of the parents (as appears to be the case in pregnant female mice faced with a foreign male). The fetus is nature's transplant; it carries antigens shared with adult lymphocytes which, by circulating in the mother's bloodstream, confront her with an immunological challenge<sup>28,30</sup>. It has been suggested that this challenge is necessary for a successful pregnancy as the maternal response induced by the implanting trophoblast blocks fetal antigens and protects the fetus from a later attack by the mother's lymphocytes<sup>31</sup>. Couples with many cell-surface antigens in common have a high frequency of spontaneous abortion, perhaps because the father shares too many cell-surface antigens with his partner to allow the fetus to elicit an early immune response strong enough to prevent a later attack by maternal cells<sup>32</sup>. When three women who had suffered several spontaneous abortions (and who shared many histocompatibility antigens with their husbands) were treated when pregnant with a mixture of foreign lymphocytes likely to provide a powerful early immune response, each had a healthy baby<sup>33</sup>. This suggests that a lack of antigenic stimulus may indeed have been responsible for their earlier failures of pregnancy. As close relatives are more likely to share cell-surface antigens, this mechanism will ensure that crosses between kin will tend to fail early in development, making the female available to a new mate.



The role of cell-surface antigens in the promotion of outbreeding may have an ancient origin. In *Botryllus*, a colonial ascidian, a system of cellular antigens controls the ability of colonies to fuse. This ability is analogous to the acceptance or rejection of foreign tissues in mammals and is controlled by an equivalently polymorphic system of self-compatibility alleles<sup>34,35</sup>. The somatic cell cell-recognition system in *Botryllus* also influences patterns of sexual reproduction, as fertilization between individuals from within the same colony (who share the same compatibility alleles) usually fail. The vertebrate histocompatibility complex may have evolved from such a self-not self sexual recognition mechanism in the early chordates.

The major histocompatibility complex has of course a profound influence on fitness through its role in resistance to disease<sup>36</sup>. Population genetic models of sexual selection suggest that a balance between mating success and other components of fitness will lead to the evolution of genetic polymorphism<sup>37</sup>. More generally, polymorphism will evolve in all genetic systems which promote outcrossing, as any newly arising allele will be favoured when rare as it is perceived as 'not kin' by most members of the population. Sexual selection may not be the entire answer to cell-surface polymorphism, but you could say that population genetics has started to smell a mouse. □

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## Cell biology

# Membrane skeleton of skeletal muscle

from Kuan Wang

To many cell biologists trying to understand how cytoskeletal filaments interact with cell surfaces, skeletal muscle cells seem to be less attractive than popular non-muscle membrane systems such as the erythrocyte membrane skeleton<sup>1</sup>, the brush border of intestinal epithelial cells<sup>2</sup> and the adhesion plaques of cultured cells<sup>3</sup>. It is therefore refreshing to see that the recent search in skeletal muscle for vinculin and spectrin — two key actin-binding proteins implicated in the attachment of actin microfilaments to the plasma membrane in non-muscle cells — has turned up something of a revelation and has added a new element to a vintage system of motility research.

Vinculin, a 130,000-molecular-weight actin-binding protein (or a 152,000-molecular-weight close relative<sup>4,5</sup>) isolated from smooth muscle and non-muscle cells, has been in the spotlight ever since its discovery four years ago<sup>6-8</sup>. In cells, it is found predominantly where actin microfilament bundles terminate at the plasma membrane, for example adhesion plaques of cultured cells, junctional complexes of epithelial cells, the dense plaques of smooth muscle cells and fascia adherens of cardiac muscle cells<sup>9</sup>. In solution, it interacts with actin, probably by binding to the ends of F-actin filaments<sup>10,11</sup>. Because of these and other attractive properties, vinculin is a prime candidate for the role of directly attaching actin bundles to specialized domains on the cytoplasmic side of the plasma membrane.

Vinculin was not however thought to be present in adult skeletal muscle, perhaps because these cells have no analogous membrane patches. This is partly why the recent paper of Padro *et al.*<sup>12</sup> has come as something of a surprise. These authors report that not only is vinculin present in skeletal and cardiac muscle, but it is organized into a striking zebra-patterned surface lattice enveloping the entire cell. What is remarkable is that the disposition and fine structure of the vinculin-containing surface lattice seems to be intimately related to that of the underlying sarcomeres in the cytoplasm. The rib-like transverse bands of the lattice, which the authors aptly dub 'costameres', encircle the muscle fibre in rolls of doublets which flank Z lines and have approximately the same width as the underlying I bands. Interestingly, the width of costameres in stretched and contracted muscle broadens and narrows as the width of underlying I-bands, as if these two types of striation are somehow coupled.

These interesting findings take on added

significance when considered together with an independent observation<sup>13</sup> that spectrin — the major actin-binding protein in the membrane skeleton of erythrocytes and many other cells — too is associated with a similar orthogonal surface lattice in skeletal muscle cells. Moreover, the transverse spectrin-containing bands which encircle the fibre also exhibit the same periodicity as the underlying sarcomeres.

Although many structural details remain to be filled in, what has now emerged is the general picture that on the cytoplasmic side of the plasma membrane of skeletal (and cardiac) muscle cells, there is a cross-striated membrane skeleton containing two distinct types of actin linker — vinculin and spectrin — in periodic arrays. Considering the mechanical stress the cell surface must endure during muscle activity, it is not unexpected that a membrane skeleton exists to provide, along with the elastic extracellular matrix, some support to the plasma membrane. The really thought-provoking observations are the striation and its coupling with the myofibril, for *a priori* there is no reason why a membrane skeleton should be striated, even for striated muscle cells. If indeed Padro *et al.*<sup>12</sup> are right, and the costamere represents sites of coupling between membrane skeleton and the myofibril, then membrane striation may arise because of the need for vinculin (or spectrin) to interact with the sarcomeric actin.

There are alternative possibilities however. It is known that skeletal muscle cells contain a cytoplasmic isoform of actin that is exclusively associated with plasma membrane and internal membrane<sup>14</sup> and is distinct from the sarcomeric isoform. If this membrane actin is part of the costamere, then vinculin may not be linked

1. Greenwood, P.J. *et al.* *Nature* **271**, 52 (1978).
2. Packer, C. *Anim. Behav.* **27**, 1 (1979).
3. Pusey, A.E. *Anim. Behav.* **28**, 543 (1980).
4. Hayashi, S. & Kimura, T. *Anim. Behav.* **31**, 81 (1983).
5. Dewsbury, D.A. *Psychol. Bull.* **89**, 464 (1981).
6. Getz, W.M. & Smith, K.B. *Nature* **302**, 147 (1983).
7. Partridge, L. *Nature* **283**, 290 (1980).
8. Breed, M.D. *Anim. Behav.* **31**, 86 (1983).
9. Michener, C.D. *Bull. ent. Soc. Am.* **28**, 7 (1982).
10. Spiess, E.B. *Am. Nat.* **119**, 675 (1982).
11. Partridge, L. & Gardner, A. *Am. Nat.* (in the press).
12. Anthony, C. & Fallon, J.-M. *J. Insect Physiol.* **28**, 873 (1982).
13. Stoddart, D.M. *The Ecology of Vertebrate Olfaction* (Chapman & Hall, London, 1980).
14. Bronson, F.H. in *Pheromones* (ed. Birch, M.C.) 345 (North-Holland, Amsterdam, 1974).
15. Berger, J. *Nature* **303**, 59 (1983).
16. Kalmus, H. *Anim. Behav.* **3**, 25 (1955).
17. Goldstein, N.I. & Cagan, R.H. in *Biochemistry of Taste and Olfaction* (eds Cagan, R.H. & Kane, M.R.) 93 (Academic, New York, 1981).
18. Buckle, G.R. & Greenberg, L. *Anim. Behav.* **29**, 802 (1981).
19. Huck, U.W. & Banks, E.M. *Anim. Behav.* **28**, 1046 (1980).
20. Lacy, R.C. & Sherman, P.W. *Am. Nat.* **121**, 489 (1983).
21. Yamazaki, K. *et al.* *Immunogenetics* **6**, 253 (1978).
22. Mainardi, D. *et al.* *Atti. Soc. It. Sci. Nat.* **104**, 325 (1965).
23. Yamazaki, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **79**, 7828 (1982).
24. Lenington, S. *Anim. Behav.* **31**, 325 (1983).
25. Yamazaki, K. *et al.* *Science* **221**, 186 (1983).
26. Yamazaki, K. *et al.* *J. exp. Med.* **144**, 1324 (1976).
27. Drife, J.O. *Br. med. J.* **286**, 294 (1983).
28. Hogarth, P.J. *Immunological Aspects of Mammalian Reproduction* (Blackie, Glasgow, 1982).
29. Lipinski, M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **78**, 5147 (1981).
30. O'Sullivan, M.J. *et al.* *Clin. exp. Immun.* **48**, 279 (1982).
31. Beer, A.E. & Sio, F.J. *Biol. Reprod.* **26**, 15 (1982).
32. Beer, A.E. *et al.* *Am. J. Obst. Gynaecol.* **141**, 987 (1981).
33. Taylor, C. & Faulk, W.P. *Lancet* **ii**, 68 (1981).
34. Scofield, V.L. *et al.* *Nature* **294**, 499 (1982).
35. Watanabe, H. & Taneda, Y. *Am. Zool.* **22**, 775 (1982).
36. Boyse, E.A. *et al.* *Hum. Immun.* **3**, 177 (1983).
37. O'Donald, P. *Theor. Pop. Biol.* **23**, 64 (1983).

1. Baines, A.J. *Nature* **301**, 377 (1983).
2. Harris, H. *Nature* **302**, 106 (1983).
3. Avnur, Z., Small, J.V. & Geiger, B. *J. Cell Biol.* **96**, 1622 (1983).
4. Feramisco, J.R., Smart, J.E., Burridge, K., Helfman, D.M. & Thomas, G.P. *J. biol. Chem.* **257**, 11024 (1982).
5. Siliciano, J.D. & Craig, S.W. *Nature* **300**, 533 (1982).
6. Geiger, B. *Cell* **18**, 193 (1979).
7. Geiger, B., Tokuyasu, D.T., Dutton, A.H. & Singer, S.J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4127 (1980).
8. Feramisco, J.R. & Burridge, K. *J. biol. Chem.* **255**, 1194 (1980).
9. Geiger, B. *Cold Spring Harb. Symp. quant. Biol.* **46**, 671 (1982).
10. Jockusch, B.M. & Isenberg, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3005 (1981).
11. Wilkens, J.A. & Lin, S. *Cell* **28**, 83 (1982).
12. Padro, J.V., Siliciano, J.D. & Craig, S.W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1008 (1983).
13. Repasky, E.A., Granger, B.L. & Lazarides, E. *Cell* **29**, 821 (1982).
14. Lubit, B.W. & Schwartz, J.H. *J. Cell Biol.* **86**, 891 (1980).
15. Pierbon-Bornoli, S. *J. Muscle Res. Cell Motility* **2**, 401 (1981).
16. Street, S.F. *J. cell. Physiol.* **114**, 346 (1983).
17. Fischman, D.A. *Curr. Top. dev. Biol.* **5**, 235 (1970).

to sarcomeric actin in the myofibril. Additionally, a direct costamere-myofibril coupling need not be invoked to explain the data of Padro *et al.* It is equally plausible that the observed coupling of two striations may arise from the presence of transverse filamentous linkages between plasma membrane and Z- and M-lines of the underlying sarcomere; and that the observed stretchiness of the costamere may arise from its close association with the elastic extracellular matrix. This alternative explanation, however, leaves open the question of why the vinculin-containing costamere is striated, and why it overlies the I band.

The significance of the striated membrane skeleton in skeletal muscle cells goes beyond support for the membrane. It may have an important bearing on muscle development. It has been known for a long time that during muscle development, before myofibril assembly, nascent sarcomeres are initiated and attached to electron-dense patches of the plasma membrane of the myotubes<sup>17</sup>. The existence of sarcomere attachment and assembly sites

on the inner surface of the membrane has always been assumed, but the molecular nature remains mysterious. In light of the presumed role of vinculin in nucleating the assembly of sarcomere-like microfilament bundles in fibroblasts, it will be very interesting to see when and where vinculin is expressed during myofibrillogenesis of developing muscle. In particular, the order and manner in which the striations of the membrane skeleton and of the sarcomere appear may shed light on the important question of whether the striated membrane skeleton directs the formation of striated sarcomeres, or vice versa. Similarly, in light of the role of spectrin in modulating the dynamic distribution of cell-surface receptors which are important in cell-cell interactions<sup>1</sup>, the expression of spectrin-containing membrane striation may be significant in understanding fusion events during development. □

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## Ecology

# Acid precipitation and the Black Forest

from Kenneth Mellanby

REPORTS in the press and on television on the ill effects of acid rain have implied widespread damage to trees, directly caused by sulphur output from industry. But by the end of a recent international meeting\* at which no less than 50 papers were delivered on the topic of acid precipitation, it was apparent that these simplistic views were neither accurate nor supported by scientific investigation.

The first half of the meeting dealt with chemical and physical topics — an assessment of emissions, the way in which oxides of sulphur and nitrogen are transformed to acid and how these acids are dispersed and deposited on soils, vegetation and water, and the techniques used to measure these pollutants. Although much accurate information is now being collected on a world scale, comparison with earlier data is difficult, as many older figures were obtained using techniques since shown to be inaccurate.

The remainder of the papers dealt with biological topics. I was not alone in eagerly awaiting the papers by German scientists, including W. Knabe, H. Schroter, U. Arnt and W. Oblander (Institute for Ecology, Rechlinghausen), and by K.E. Rehfuess, K.D. Jung and B. Prinz (Institute for Air Pollution Control, Essen) on the situation in the German forests. Although a recent excellent paper on acid rain and forest

decline in West Germany by W.O. Binns and D.B. Redfern (British Forestry Commission; Research and Development Paper 131, 1983) had indicated that the situation was much less alarming than popular accounts had suggested, some concern is clearly well founded. The German scientists reported that damage, particularly dieback in fir (*Abies abies*) and spruce (*Picea abies*) was only local, but it was impossible not to face the possibility that it might spread rapidly.

The cause of the damage was not agreed. There were few supporters for the view that acid precipitation had been proved to be the only or even the main culprit. The general consensus suggested that the most likely cause was the combination of a cold winter, a dry summer, fungal disease, elevated ozone levels with, possibly, raised aluminium levels in the soil water arising from the effects of acid precipitation. However there seemed little support for the views of B. Ulrich (Institute of Soil Science and Forest Nutrition, Göttingen) that, at present, this effect of precipitation on soil aluminium is the major cause of damage to trees in Germany. Elsewhere, as in Scandinavia, reports of forest destruction have clearly been serious overestimates.

It is generally accepted that many rivers and lakes, particularly where the water has little buffering capacity, have become more acidic in the last thirty years, and that fisheries have been seriously affected. The reasons for the increased acidity appear to

be complex. I. Th. Rosenquist (University of Oslo), as a result of work on ion exchange in soil and fresh water, queried many of the generally accepted results, and showed that acidification was often dependent on changes in land use rather than on precipitation. He showed that the phenomenon of acidification was not new, and incidents had occurred long before the output of oxides of sulphur and nitrogen from industry reached the present levels.

In my opinion some of the speakers still failed to distinguish between the effects of dry deposition of sulphur dioxide, and the deposition of acidic substances in precipitation. The distinction was clearly demonstrated by S. Luckat (University of Dortmund), who spoke on the effects on materials, stonework in particular. Here dry deposition by SO<sub>2</sub> in cities has long been known to cause serious deterioration, while acid rain has had little effect. This is partly because the concentration of pollutants often falls as the period of precipitation continues — the first rain washes out much acid, the last part of the rain to fall may be comparatively pure. Thus the statues are washed clean by this rain, which may also remove some of the toxic products of dry deposition.

After the conference I took the opportunity of a conducted tour of the Black Forest. This was a revealing experience. Far from the widespread devastation highlighted in a recent television programme shown in Britain, the ordinary tourist, or even the visiting scientist, could travel for days without seeing any serious damage. There are indeed damaged areas, and we were taken to some of them. Even here, it was generally individual trees that were affected, and, when asked, our guides were unable to demonstrate a single tree that had actually died recently. That is not to deny that some are probably doomed, and the rapid development of symptoms in spruce, a species previously relatively immune from dieback, is clearly worrying. The damage seems to be confined to high altitudes, where the trees, growing near the tree line, must be under stress.

One thing is certain. The damage in the Black Forest cannot be the result of SO<sub>2</sub> emissions in the Federal German Republic. Levels of SO<sub>2</sub> in the forest are very low indeed, as is demonstrated by the wealth of foliose and leafy lichens, particularly on moribund firs. If sulphuric acid is to blame, the time taken for its production in the atmosphere is such that its origin must be more distant. So the policy of the German government, spurred on by the Green Party, to reduce sulphur output from their industry to low levels, at a very considerable expense, may be, on a global scale, very altruistic, but it will not do any good (or any harm) to the trees of the Black Forest. □

Kenneth Mellanby is editor of Environmental Pollution and chairman of the Watt Committee on Energy's working group on acid rain.

\*An international conference on 'Acid Precipitation', organized by the Verein Deutscher Ingenieure's Commission on Air Pollution, met in Lindau, on Lake Constance, FRG from 7 to 9 June.



## Materials science

## Preparation of soluble conducting polymers

from Paul Calvert

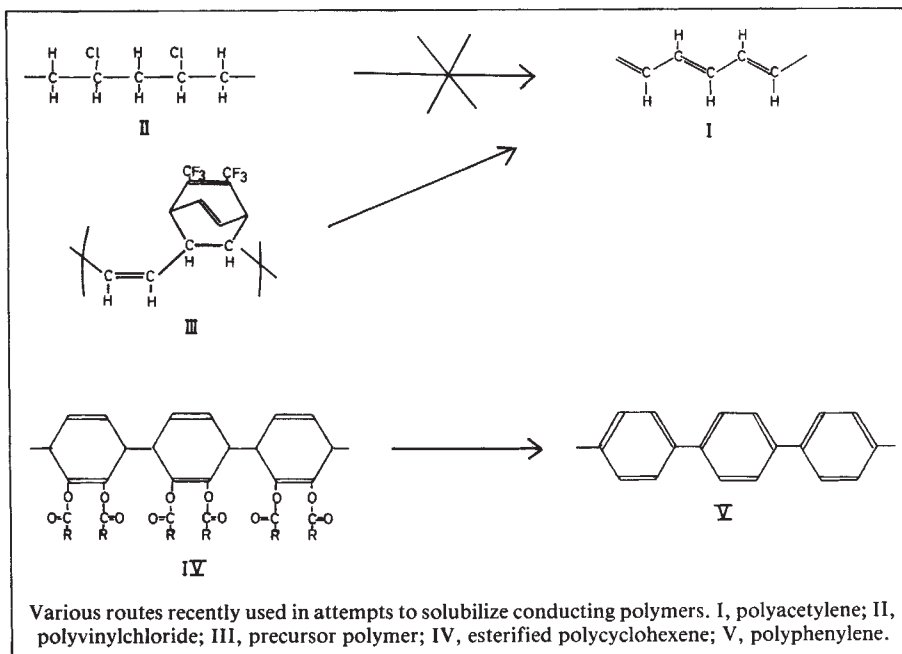
CONDUCTING polymers have been the focus of a great deal of research in the last few years. The interest started with the discovery that acetylene could be polymerized to a shiny black film, polyacetylene (I in the figure), which became highly conducting when 'doped' by reaction with large amounts of strong oxidizing or reducing agent<sup>1-3</sup>. The conductivity is attributable to the ease of electron transport along the chain. Many other polymers with similarly conjugated double bonds have been found to conduct when doped.

The promise offered by these materials is of a class of conductors or semiconductors that would carry the benefits associated with polymers: cheapness, toughness and easy processing to fibres, films and coatings on a large scale. Unfortunately most of these convenient properties are lost in the conjugated systems whose chain rigidity and strong interchain forces make them insoluble infusible black powders. Recent months have seen the publication of a number of routes to solubilizing conducting polymers (see the figure).

One route is to prepare a flexible soluble precursor polymer which can be readily cast into films before conversion to polyacetylene. In principle, polyvinylchloride (PVC; II in the figure) should do this because it is degraded on heating by loss of HCl to leave a black polyacetylene-like material. However the result is not conducting when doped, apparently because removal of adjoining H, Cl pairs at random will leave many isolated Cl or H atoms which break up the conjugated sequences. A way of avoiding this is to pair up the leaving groups beforehand. Jim Feast and his co-workers have produced a number of precursor polymers which lose cyclic compounds on heating and transform to polyacetylene<sup>4,5</sup>. The unnameable precursor polymer (III) is soluble in acetone and forms transparent films which, at 50–80°C, lose hexafluoro-*o*-xylene and 70 per cent of their weight to leave black shiny dopable amorphous polyacetylene. Interest is centring on the fact that this material is different in structure and properties from polymer prepared by direct polymerization, but characterization of black insoluble films is no easy task.

Another approach to solubility is to modify the polymer to build enough flexibility into the chains to allow them to dissolve without losing conductivity. The results of copolymerization with small amounts of polymethylacetylene have been disappointing because conductivity rapidly decreased as side groups were added to the chain.

Workers at Bell Laboratories now report graft copolymers with acetylene chains of



20,000 molecular weight attached to polyisoprene<sup>6</sup>. These soluble polymers have a separate acetylene phase in the solid state and yield interesting spectroscopic data but there is no mention of conductivity. Galvin and Wnek<sup>7</sup> have described previously a similar system where acetylene is polymerized inside polyethylene to produce a conducting composite.

Polyacetylene has the disadvantage that it is not very stable to oxidative degradation. From this point of view polyphenylene is a much more attractive polymer but it has only been available so far as an intractable powder of very short chain length. Ballard and co-workers at ICI Runcorn are now reporting<sup>8</sup> a route through a soluble precursor polymer, an esterified polycyclohexene (IV) which converts to polyphenylene (V) on heating to 240°C.

This will be of great interest because the polyphenylenes studied to date have been poorly characterized materials with short chain lengths and it offers the exciting prospect of being able to control the chain length and composition and study the effect on properties. An added bonus is that there is a bacterial fermentation route from benzene to the precursor. This may not materially affect the final polymer but it has enabled Ballard and colleagues to perform the remarkable feat of joining both the biotechnology and conducting polymer bandwagons at once.

Recently published studies on another of these polymers, polyphenylenesulphide, at Allied Chemical, illustrates the complexity of solubilizing them<sup>9</sup>. Strange effects were found when this polymer was doped with

arsenic pentafluoride contaminated with arsenic trifluoride. AsF<sub>3</sub> had little effect on the polymer itself but seemed to be a strong plasticizing agent for the AsF<sub>5</sub>-doped state. Using liquid AsF<sub>3</sub> Frommer and his colleagues now report that the doped polymer dissolves to a blue solution from which they can cast tough conducting films. When cast the film will not redissolve because of cross-linking. It seems often to be the case that such powerful dopants also cause side reactions and cross-linking.

This year a mood of realism has been spreading over this rapidly growing field. Various applications for conducting polymers have been proposed in the past, including solar cells, semiconductor devices and batteries, but these are still a long way off and a polymeric replacement for copper wires looks unlikely. Those interested in silicon semiconductors have been pointing out that the polymers are impure unstable uncharacterized junk compared with single-crystal silicon. The availability of soluble materials that can be purified and characterized should improve this state of affairs. □

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1. See Calvert, P. *Nature* **284**, 213 (1980).
2. Wegner, G. *Angew. Chem. Int. Edn* **20**, 361 (1981).
3. Baughman, R.H. *et al. Chem. Rev.* **82**, 209 (1982).
4. Edwards, J.H. & Feast, W.J. *Polymer* **21**, 595 (1980).
5. Feast, J. *Polymer* (submitted).
6. Bates, F.S. & Baker, G.L. *Macromolecules* **16**, 704 (1983).
7. Galvin, M.E. & Wnek, G.E. *Polymer* **23**, 795 (1982).
8. Ballard, D.G.H., Courts, A., Shirley, I.M. & Taylor, S.C. *Chem. Commun.* (submitted).
9. Frommer, J.E., Elsenbaumer, R.L. & Chance, R.R. *ACS ORPL Preprints* **48**, 552 (1983).

## Astronomy

## Emission lines from galactic nuclei

from B.E.J. Pagel and M.G. Edmunds

QUASARS, radio galaxies, Seyfert galaxies and many other galaxies display a variety of emission-line spectra from their nuclei. These spectra have some features in common with the relatively well understood spectra of planetary nebulae, HII regions and supernova remnants, but there are also significant differences in line profiles and intensity ratios.

Even galaxies usually regarded as 'normal', such as the nearby spirals M51 and M81, have long been known to have nuclei in which a faint emission-line spectrum is superimposed on the underlying continuum and absorption lines of a stellar population. The development of linear digital detectors capable of high spatial and spectroscopic resolution at low light levels has revealed many more than these 'low-ionization nuclear emission-line regions' (acronym 'liners') which are characterized by an H $\gamma$  line with broad wings and remarkably strong [NII] lines on either side. In 1981, T.M. Heckman, now at the University of Maryland, found at least 30 per cent of large spiral galaxies to have this type of emission; more recently W.C. Keel at the Lick Observatory, Santa Cruz, has shown that virtually all of them do, at least in those cases that are not dominated by some other kind of emission (*Astrophys. J.* **269**, 446; 1983). J.A. Baldwin and M.M. Phillips of Cerro Tololo Inter-American Observatory, Chile, and R. Terlevich of the Institute of Astronomy, University of

Cambridge, showed in 1981 that the line intensity ratios in the various types of object — planetaries, HII regions, narrow-line components of Seyfert galaxies and liners — follow quite separate patterns indicating distinct excitation and ionization mechanisms in the galactic nuclei; but what are these mechanisms? This question was the main topic of a recent meeting\*.

Quasars and Seyfert 1 galaxies, which have a smooth non-stellar continuum, broad hydrogen and helium lines and narrow forbidden lines, are strong X-ray sources and probably both the broad- and the narrow-line-emitting regions are photoionized by a hard spectrum which can be represented (in part, at least) by a power law  $F_\nu \sim \nu^{-1.5}$  or so. A similar photoionization mechanism applies to Seyfert 2 galaxies where narrow lines dominate the spectrum. The penetration of X rays into predominantly neutral zones adjoining ionized material nearer to the radiation source causes emission from neutral species such as [OI] and contributes emission to the hydrogen lines. For liners, which are often associated with compact radio sources, the situation is more ambiguous. Photoionization models had in the past failed to predict strong enough [OI] and [NII] and so people had turned to shock models similar to those which are

\*A workshop on 'Ionization mechanisms in emission-line nuclei of galaxies', was held at the Royal Greenwich Observatory, Herstmonceux, on 6–7 April 1983.

successful in accounting for supernova remnants. However, improved photoionization models incorporating the effects of charge-exchange reactions between ionized and neutral atoms have now been shown by D. Péquignot (Observatoire de Meudon), G. Ferland (University of Kentucky) and H. Netzer (Tel Aviv University; *Astrophys. J.* **264**, 105; 1983) and by J. Halpern (Harvard Center for Astrophysics) and J.E. Steiner (Universidade de São Paulo; *Astrophys. J. Lett.* **269**, L37; 1983) to give an account of liner spectra as good as, and in some respects better than, that provided by the shock models, given the assumption of a spectral distribution similar to that in Seyferts but with a more diluted radiation field relative to the particle density.

A potentially crucial test — measurement of the electron temperature in the more ionized zone from the ratio of the weak and strong lines  $\lambda\lambda 4363$  and 5007 of [OIII] — unfortunately gives ambiguous results because of the masking effect of the complex absorption spectrum of the stellar background. Several plausibility arguments (such as continuity with the less luminous Seyfert galaxies) now support the photoionization models, but M.A. Dopita (Mount Stromlo and Siding Spring Observatories, Australian National University) pointed out the potentially interesting consequences of very high-velocity shocks leading to X rays, and there were some reservations about the explanation of the various iron-line spectra ranging from FeII to [FeXIV]. Interesting information on the details of excitation mechanisms (and perhaps evidence of more exotic plasma processes) is likely to spring from the observations by Heckman and his colleagues of emission-line spectra that are spatially associated with jets of radio emission in or near active nuclei.

Various problems and possibilities are raised by the line profiles. In quasars the widths of lines (which measure the velocity field in regions where the lines are formed) are remarkably similar for species of widely differing excitation potential that might have been expected to be widely stratified in space, provoking G. Shields (University of Texas at Austin) to put forward an unconventional model in which the broad lines come from the surface of a rapidly rotating superstar or spinar excited by radiation from sources extended along the polar axis. On the other hand, A. Filippenko (Cal Tech) and J.A. Baldwin had studied cases of active galaxies in which the line widths were found to increase systematically with the theoretical critical density at which collisional de-excitation becomes equally probable with radiative de-excitation and therefore begins to quench the line, indicating a clear stratification of the velocity field with depth.

Another sidelight is cast on ionization mechanisms by studies of variability over a wide range of wavelengths, notably the systematic monitoring programme of the

## 100 years ago

THE SUPPOSED HUMAN FOOTPRINTS  
RECENTLY FOUND IN NEVADA

DURING the past summer various accounts have been published of the discovery of human footprints in sandstone near Carson, Nevada. Many different kinds of tracks were found, some of which were made by an animal allied to the elephant; some resembled those of the horse and the deer; others were apparently made by a wolf. There were also tracks made by large birds.

The supposed human footprints are in six series, each with alternate right and left tracks. The stride is from two and a half to over three feet in extent. The individual footprints are from eighteen to twenty inches in length, and about eight inches wide. The distance between the line of right-hand and left-hand tracks, or the straddle, is eighteen to nineteen inches.

The form and general appearance of the supposed human tracks is shown in Fig. 2. The size of these footprints, and especially the width between the right and left series, are strong evidence that they were not made by men, as has been so generally supposed.

A more probable explanation is that the impressions are the tracks of a large sloth, either *Myiodon* or *Morotherium*, remains of which

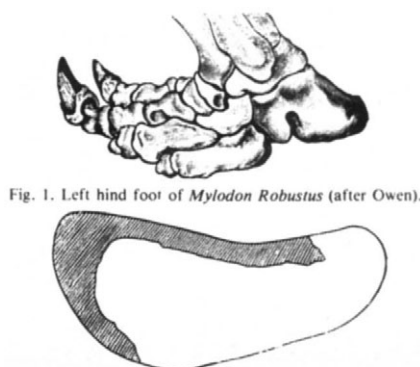


Fig. 1. Left hind foot of *Myiodon Robustus* (after Owen).

Fig. 2. Left footprint at Carson (after Harkness).

have been found in essentially the same horizon. In support of this view it may be said that the footprints are almost exactly what these animals would make if the hind feet covered the impressions of those in front. In size, in stride, and in width between the right and left series of impressions, the footprints agree closely with what we should expect *Myiodon* or *Morotherium* to make (Fig. 1).

The geological horizon of these interesting footprints is near the junction of the Pliocene and Quaternary.

From *Nature* **28**, 370, 16 August 1883.



Seyfert 1 galaxy NGC4151 by a large inter-natal group. A. Snijders (Royal Greenwich Observatory) described the results obtained so far, which suggest the presence of two distinct components in the radiation field: a relatively constant component resembling a black body at 30,000K and a variable power-law component which is manifested in the variability of broad high-excitation lines over periods of weeks.

In HII regions and planetary nebulae, it is safe to assume that the intrinsic relative intensities of hydrogen lines are given by the classical 'case B' recombination theory which assumes that all line photons from transitions to the ground state are completely absorbed with the exception of Lyman- $\alpha$  whereas all other line photons freely escape; thus from a comparison with the intensity ratios actually measured one can deduce the amount of reddening and extinction by interstellar dust. To what extent can one make the same assumption in galactic nuclei? This question was addressed from different viewpoints by J. Bergeron (Institut d'Astrophysique, Paris), C.M. Gaskell (Institute of Astronomy, Cambridge), J. Halpern, A. Lawrence (Royal Greenwich Observatory) and D.E. Osterbrock (University of California, Santa Cruz). X-ray heating of the neutral zones causes additional excitation of hydrogen lines leading often to relative intensities described by a 'case B' which differs from case B but not by very much. Thus it seems that dust-reddening makes a substantial contribution (though by no means the only one) to the large H $\alpha$ /H $\beta$  ratios often observed in active galactic nuclei.

Finally, R. Terlevich (University of Cambridge) and D.W. Weedman (Pennsylvania State University) pointed out that rapid star formation is itself a form of activity which is seen proceeding on an enormous scale in blue compact galaxies and in the immediate surroundings of hot-spot and starburst nuclei where it is associated in some cases with Seyfert or liner spectra coming from the nucleus itself. The relationship between such relatively conventional star-forming activity and the more exotic mechanisms operating in galactic nuclei has been interpreted by Weedman (*Astrophys. J.* **266**, 479; 1983) as a sign that star formation leads to a cluster of black holes of stellar mass which then cause non-thermal excitation. However, the time scale of variability, the confinement to nuclei and other difficulties with this picture lead many workers to prefer the canonical model of a single black hole with  $10^8$  or more times the mass of the Sun that may be more or less 'active' according to the rate at which it can swallow fresh material from its surroundings. Future work may help to clarify the relationship between this type of activity and violent star formation. □

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## Neurophysiology

# Listening with one cell

from Jonathan Ashmore

THE cochlea in the inner ear of mammals is capable of detecting sounds whose frequencies vary over seven orders of magnitude — a feat whose mechanism is still not understood. It has however become clear that the extraction of frequency information in non-mammalian vertebrates often depends on inner ear structures somewhat different from those of mammals, and operating on rather different principles. Although the frequency range detectable by lower vertebrates is more restricted than the mammalian one, the hope is that the transduction mechanism by which the sensory cells convey frequency information to the auditory nerve will prove to be the same<sup>1</sup>. The paper by Lewis and Hudspeth in this issue of *Nature*<sup>2</sup>, describing the membrane properties of frequency-sensitive hair cells in the frog, may thus have general significance for understanding how lower vertebrates convey frequency information to the auditory nerve.

In mammals, the basis of frequency discrimination is believed to be the mechanical travelling wave set up in the inner ear by the sound wave and first described by von Békésy<sup>3</sup>. The wave travels along the basilar membrane on which the hair cells rest and the point in the membrane at which the wave reaches its peak amplitude depends on the frequency of the sound. This mechanically ensures that different acoustic frequencies stimulate distinct sets of cochlear hair cells. Improved experimental methods have shown that the basilar membrane tuning is sharper than can be accounted for by its passive mechanical properties alone<sup>4,5</sup> and the emphasis has shifted to those properties of the hair cells and the structure of the cochlear partition that could be responsible. The details remain as enigmatic as ever but recent modelling attempts<sup>6</sup> have suggested that energy has to be fed into the basilar membrane in the region of the envelope peak in order to account for the observed tuning.

In some species tuning can occur even in the absence of a travelling wave. Where the dividing line occurs is blurred, but in the caiman crocodile there may only be a poorly developed travelling wave system to detect sounds with frequencies of up to about 3 kHz (ref. 7). Some lizards possess a population of hair cells where the stereocilia are free-standing in endolymphatic fluid instead of being attached to the tectorial membrane, as in mammals. In this case each hair cell seems to respond to frequencies determined by the micro-mechanics and oscillation pattern of its stereocilia<sup>8</sup>.

The mechanism that operates in turtle auditory papilla is less equivocal. Although the turtle basilar membrane is not place-

tuned, each cell responds maximally to one frequency over the working range of 70–670 Hz (ref. 10), as a result of an electrical resonant filter arising from properties of the cell membrane probably distinct from the transduction site. This type of tuning is likely to be used in other mechanoreceptive organs<sup>11,12</sup> and there are hints that electrical resonance operates in fish electroreceptors<sup>13</sup>.

More uncertain is the case of the bird cochlea which has both basilar and tectorial membranes as has the mammal. However, the spontaneous pattern of activity in the auditory nerve fibres shows a periodicity<sup>14</sup> which, as in the turtle<sup>10</sup> and gecko<sup>15</sup>, suggests that the hair cells have a tuning mechanism which depends on an electrical rather than mechanical separation of frequency. With up to 54 hair cells per row in some birds as opposed to the 4 frequently found in mammals, the avian cochlea is an obvious target for the keen electrophysiologist with a sharp electrode.

When current is injected into turtle hair cells, electrical tuning is revealed as a damped oscillation of the membrane potential at a frequency coinciding with the cell's best acoustic frequency<sup>10</sup>. It has long been known that excitable membranes are capable of producing subthreshold oscillatory responses near 100 Hz when K<sup>+</sup> and Na<sup>+</sup> carry the ionic current<sup>16</sup>. Lewis and Hudspeth have now addressed the question of the identity of the channels that may be involved in the hair-cell resonance. By working on isolated hair cells of the frog sacculus, and using patch electrode techniques to voltage-clamp and dialyse internally the cells, they report the presence of three ionic currents activated near the resting membrane potential. In different cells they have demonstrated a potassium

- Hudspeth, A.J. *A. Rev. Neurosci.* **6**, 187 (1983).
- Lewis, R.S. & Hudspeth, A.J. *Nature* **304**, 538 (1983).
- Von Békésy, G. *Experiments in Hearing* (McGraw-Hill, London, 1960).
- Khanna, S.M. & Leonard, D.G. *Science* **215**, 305 (1982).
- Selick, P.M., Patuzzi, R. & Johnstone, B.M. *J. acoust. Soc. Am.* **72**, 131 (1982).
- de Boer, E. *J. acoust. Soc. Am.* **73**, 567 (1982).
- Klinke, R. & Pause, M. *Expl. Brain Res.* **38**, 137 (1980).
- Weiss, T.F. *et al.* in *Evoked Electrical Activity in the Auditory Nervous System* (eds Naunton, R.F. & Fernandez, C.) 91 (Academic, New York, 1978).
- Frishkopf, I.S., DeRosier, D.J. & Egelman, E.H. *Soc. Neurosci. Abstr.* **8**, 40 (1982).
- Crawford, A.C. & Fettiplace, R. *J. Physiol., Lond.* **312**, 377 (1981).
- Ashmore, J.F. *J. Physiol., Lond.* **336**, 24P (1983).
- Lewis, R.S. *Soc. Neurosci. Abstr.* **8**, 728 (1982).
- Meyer, J.H. & Zakon, H.H. *Science* **217**, 635 (1982).
- Manley, G.A. *Naturwissenschaften* **66**, 582 (1979).
- Eaton, R.A., Manley, G.A. & Pawson, L. *J. comp. Physiol.* **142**, 203 (1980).
- Hodgkin, A.L. & Huxley, A.F. *J. Physiol., Lond.* **117**, 500 (1952).
- Bader, C.R., Bertrand, D. & Schwartz, E.A. *J. Physiol., Lond.* **331**, 253 (1982).
- Lewis, E.R., Leverenz, E.C. & Koyama, H. *J. comp. Physiol.* **145**, 37 (1982).

current, similar to an inactivating current  $I_A$  described in molluscan neurones; a calcium current,  $I_{Ca}$ ; and a calcium-dependent potassium current,  $I_{K(Ca)}$ . These currents are known from other preparations, but a novel feature of the  $I_{Ca}$  described is that it has a fast activation ( $<5$  ms) and seems not to inactivate. The sensitivity of the membrane oscillations and  $I_{K(Ca)}$  to TEA<sup>10</sup> suggests that at least one of the currents underlying this type of tuning may have been pinned down. A similar pharmacological dissection technique has been used to uncover ionic mechanisms in photoreceptors, where, at the last count, seven distinct currents have been described<sup>17</sup>. It would be surprising if the count in hair cells does not increase from three.

It remains to be shown that modelling

the interplay of these currents can account quantitatively for tuning in saccular hair cells, where resonant frequencies extend to about 160 Hz (ref. 18). Harder to answer is whether the same results apply to other hair cells where frequency-tuning occurs over a greater range. Does regulation of the internal calcium levels or the amplification produced by involving an internal messenger ion generate a wider range of possible frequencies? Most tantalizing is the developmental question: can this type of cellular mechanism be regulated to produce tonotopic organization in an auditory papilla? In contrast, perhaps von Békésy was describing the simplest engineering principle on which to build a cochlea. □

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## Neurobiology

# Transfer of information in the visual cortex

from Nicholas V. Swindale

THE paper by Livingstone and Hubel in *Nature* this week<sup>1</sup> provides a further example of the importance of cytochrome oxidase patches, first discovered by Margaret Wong-Riley in the upper layers of the primary visual cortex of primates, in disentangling the anatomical relationships between the columns of neurones that represent the functional subdivisions of the visual cortex. The importance of the patches lies in their use as anatomical markers, since they have a fixed spatial relationship with at least one and probably several other types of column, while the histological method used to demonstrate them is simple and can easily be used in combination with other methods for studying columnar structure.

There are several possible explanations for the existence of the patches. It can be assumed that increased levels of the enzyme are in the first place the result of an increased level of metabolic activity, and that this must be due either to chronically higher levels of activity in the neurones within the patches, or to the type or rate of transmitter synthesis that is going on<sup>2</sup>.

Two observations suggest that a higher level of activity is at least part of the explanation. First, the absolute levels of the enzyme within each patch are not greater than they are within layer IV, which stains heavily and uniformly. It has been shown that the patches receive a direct input from the lateral geniculate nucleus<sup>3</sup>, as does layer IV, and if activity in these terminals is the cause of the high levels of the enzyme in layer IV, it might also cause the elevated levels within the patches. Second, physiological studies show that cells in the patches lack orientation selectivity<sup>4</sup>, suggesting that they would probably respond to a

greater variety of stimuli than other cortical neurones. Many also have little or no low-spatial-frequency cut-off<sup>5</sup>, implying that they should respond to diffuse light, something that other cortical cells will not do.

Anatomically, the patches seem to be related to several different types of column. It is known that each patch is aligned with the centre of an ocular dominance stripe<sup>6</sup>, and that each contains a high proportion of cells that respond selectively to coloured stimuli<sup>7</sup>. Thus it is likely that the patches either coincide, or intersect with the columns of colour-selective cells described by Michael<sup>8</sup>. Recent work by Tootell and his colleagues<sup>9</sup> suggests that the patches may relate to other types of column as well. Deoxyglucose studies where sine wave gratings of all orientations, but only one spatial frequency, are used to stimulate the cortex suggest that spatial frequency columns are arranged concentrically around the patches, cells between the patches being tuned to higher spatial frequencies than cells within the patches. Horton's and Hubel's original observation<sup>6</sup> that the patches intersect, or lie on the borders of orientation columns has been repeated by Tootell *et al.*<sup>9</sup>, strengthening the argument that different orientation columns must coalesce in or near the patches. The overall arrangement of columns that these results suggest, with orientation columns radiating out of the patches ranged concentrically around them, makes a good deal of sense, since it would allow every combination of spatial frequency and orientation to be represented within the diameter of an ocular dominance stripe. This is an important requirement if representation of visual information by the visual

cortex is to be complete.

Livingstone and Hubel<sup>1</sup> have now taken the analysis one step further by using the cytochrome oxidase stain in conjunction with the horseradish peroxidase tracing method to study the organization of the projection from the primary visual cortex (area 17) to the neighbouring area 18. Cytochrome oxidase staining in area 18 reveals a pattern not of patches, but of short darkly staining strips, alternately thick and thin, and separated by narrow lightly staining strips<sup>10</sup>. The repeat periodicity (from one thin dark strip to the next) is about 2.5 mm, probably the largest repeat period for any columnar structure yet discovered in the cerebral cortex (suggesting that other still lower periodicities might exist in other cortical areas). Livingstone and Hubel have now shown that the patches in area 17 project specifically to the thin dark strips in area 18 (the existence of a projection to the thick dark strips is equivocal), while conversely, non-patch regions in area 17 project to the palely staining inter-strip regions.

Two of Livingstone's and Hubel's findings are worth commenting upon. First, the region of cortex in area 17 that projects to an area 18 strip is about 2–3 mm wide — substantially larger than the injected area within the strip, which is about 0.5 mm wide. Second, both left and right eye patches (those superimposed on left or right eye ocular dominance columns) participate in the projection. Thus there is an overall convergence of information — from both eyes, and from a relatively large region of area 17 — to single small regions in the area 18 strips. It is reasonable to assume that a columnar microstructure (with properties varying over distances of tens to hundreds of micrometres) exists within area 18 as it does in area 17. Just what properties might change on such a scale within the strips is not known, but they are presumably ones not possessed by cells within the area 17 patches. These two types of transformation, condensation of some features of the representation within area 17, in parallel with the elaboration and creation of new ones, may turn out to be a general feature of the way in which information is transformed in the projection from one cerebral cortical area to another. □

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- Livingstone, M.S. & Hubel, D.H. *Nature* **304**, 531 (1983).
- Hendrickson, A.E., Hunt, S.P. & Wu, J.W. *Nature* **292**, 605 (1981).
- Livingstone, M.S. & Hubel, D.H. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6098 (1982).
- Hubel, D.H. & Livingstone, M.S. *Soc. Neurosci. Abstr.* **7**, 357 (1981).
- Parker, A.J. & Hawken, M.J. *Invest. Ophthalmol. Suppl.* **22**, 11 (1982).
- Horton, J.C. & Hubel, D.H. *Nature* **292**, 762 (1981).
- Hubel, D.H. & Livingstone, M.S. *Soc. Neurosci. Abstr.* **8**, 706 (1982).
- Michael, C.R. *J. Neurophysiol.* **46**, 587 (1981).
- Tootell, R.B.H., Silverman, M.S., Switkes, E. & De Valois, R.L. *Soc. Neurosci. Abstr.* **8**, 707 (1982).
- Tootell, R.B.H., Silverman, M.S., De Valois, R.L. & Jacobs, G.H. *Science* **220**, 737 (1983).



## Evolution of oncogenes

## From c-src to v-src

from John Wyke

In a recent issue of *Nature*, Jean Wang discussed the differences between the cellular and viral versions of the *abl* oncogene<sup>1</sup>. This week John Wyke reports some recent explorations of two versions of *v-src* and its cellular homologue.

WITH the single exception of the *sis* oncogene, now known to encode a protein at least partly homologous to platelet-derived growth factor<sup>2</sup>, what we know of cellular oncogenes and their products comes largely from studies on viral oncogenes. It is therefore important, as well as interesting, to determine how viruses acquired oncogenes from their host and how this capture has affected oncogene regulation and function. With this in mind, three groups have recently undertaken comparisons of the nucleotide sequences of viruses that contain the Rous sarcoma virus (RSV) *v-src* gene with those of related viruses that lack them, and with the homologous cellular oncogene<sup>3-5</sup>. Their reports form an interesting triad of papers from which, despite discrepancies of data and interpretation, an intriguing if tentative consensus can be drawn. This provides both insights on *v-src* evolution and provocative questions for further study.

The comparisons were made between the sequences of the Prague (PR) and Schmidt-Ruppin (SR) strains of RSV and comparable regions of RAV-O, which lacks an oncogene and is thought to resemble the progenitor of RSV (see Fig.1). As expected, the transforming viruses have acquired sequences homologous to the cell oncogene, *c-src*, comprising a non-

translated 5' portion (NS) and coding sequences (TS). The cellular *src* gene however is split into several exons, whereas the viral gene is continuous, which suggests that the virus acquired the *src* gene from a spliced RNA intermediate. The RSV strains have also gained two other nucleotide tracts of unknown origin: a sequence (NA) only partially homologous in the two strains and immediately 3' to *src*, and an element (E) that is 3' to *src* in PR-RSV but 5' to it in SR-RSV. Interspersed with these foreign sequences are regions with homology to the 3' end of RAV-O RNA. A tract (D) becomes duplicated as a direct repeat either side of the *src* insert whilst a sequence (B) immediately 3' to D in RAV-O is now found thrice in each RSV strain, notably as a repeat flanking the E insert. Adjacent to B in RAV-O is a polypurine tract (thought to be important in plus-strand DNA synthesis during reverse transcription) that defines the 5' boundary of the unique 3' (U3) region common to all these viruses. Both P and the region of U3 immediately neighbouring it (O) are duplicated 5' to *src* in PR-RSV only.

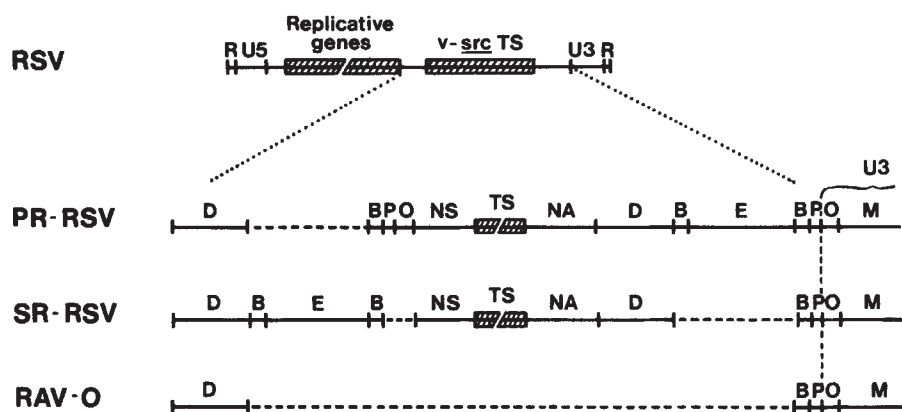
What is the reason for this variable arrangement of exotic and retroviral sequences in PR- and SR-RSV? Perhaps each strain acquired both *src* and E sequences independently, but Schwartz *et al.*

postulate the derivation of the two strains from a common transforming ancestor. Figure 2 outlines a combination of this hypothesis and the proposed mechanism for *c-src* transduction of Swanstrom *et al.* In the first stage, a non-transforming virus such as RAV-O (Fig.1) acquires an insertion element, E, which integrates at B and causes duplication of the B sequence. The proviral DNA from this novel virus then integrates fortuitously upstream of *c-src* (Fig.2a), where deletion of the 3' proviral long terminal repeat (LTR) (except the U3 sequences designated O) and perhaps of flanking cell DNA (Fig.2b) removes the LTR poly(A) addition signals and permits synthesis of hybrid RNA comprising viral and spliced *c-src* sequences. Because it still has the appropriate signals at its 5' (viral) end, this molecule can be packaged in a virion as a dimer with authentic virus genomic RNA (Fig.2c). To generate the existing RSV proviruses from this dimer, Swanstrom *et al.* postulate recombination between the two strands during reverse transcription (Fig.2c). A cross-over between a point to the left of U3 on the virus RNA and to the right of *c-src* on the hybrid RNA generates a provirus containing *src* yet retaining the terminal R, U3 and U5 regions needed for its replication. If the cross-over point on virus RNA was upstream of D (Fig.2c), then a provirus would arise in which *v-src* is flanked by a direct repeat of the sequence DBEBPO. Schwartz *et al.* point out that differential deletion by recombination at regions of homology in this hypothetical ancestral RSV (broken lines in Fig.1) can yield the arrangement of sequences seen in each RSV strain.

This model makes predictions that are supported by some features of the RSV structure. First, recombination between the left end of *c-src* and viral sequences should have occurred at the DNA rather than the RNA level (Fig.2a,b). This is reflected in the point of recombination, which is within an intron of *c-src* immediately upstream of a transduced 5'-noncoding exon (this exon provides a splice acceptor site used to produce both *v-src* and *c-src* mRNA).

Second, later stages of *c-src* transduction should have involved processed RNA transcripts (Fig.2c). Consonant with this is the absence of introns in *v-src*.

However, a further prediction, that the right-hand recombination between *c-src* and virus must have occurred in a *c-src* exon (Fig.2c), has not been validated because the origins of the 3' terminus of *v-src* are uncertain. Takeya and Hanafusa have probably determined correctly the 3' end of the *c-src* coding sequence (a cDNA clone of the gene is needed to confirm this) and it encodes 19 amino acids that differ from the corresponding 12 amino acids encoded by the 3' terminus of *v-src*. Most of this *v-src* region (the last few nucleotides are not accounted for) seems to derive from a cell sequence, about 900 base pairs downstream



**Fig.1** Comparison of RNA sequences towards the 3' end of the genomes of PR-RSV, SR-RSV and RAV-O. The top diagram depicts the whole genome of RSV and locates the regions under discussion. Lettering: Different symbols were used for equivalent regions in the papers under discussion; the lettering used here does not, in most cases, coincide with that in any of the papers. R, a sequence repeated directly at each end of viral RNA; U3, sequence unique to the 3' end of viral RNA; U5, sequence unique to the 5' end of viral RNA. U3-R-U5 together form the long terminal repeat, formed at each end of proviral DNA, that contains elements regulating virus expression and replication; D, a sequence present in RAV-O that is directly repeated either side of *src* in RSV; B, sequence present in RAV-O, the presumed target site for insertion of E, an element of cell origin acquired by RSV; P, a polypurine tract defining the 5' boundary of U3; O, the 5' portion of U3, adjacent to P; M, the remainder of U3; NS, a noncoding sequence derived from the *c-src* locus; TS, the coding sequence of *v-src*, largely derived from *c-src*; NA, a sequence of unknown origin immediately 3' to *v-src*.

from the *c-src* termination codon. This region is not detectably transcribed, so Takeya and Hanafusa conclude that its linkage with *src* occurred by recombination at the DNA level. If this conclusion and the model in Fig. 2 are both correct they can be reconciled by postulating that transforming virus was only acquired by recombination with a rearranged *c-src* gene that forms an abnormal transcriptional unit.

The origin of the non-attributed (NA) sequence immediately 3' of the *v-src* coding region is also an enigma. This sequence is in the region of the proposed cross-over during reverse transcription and, significantly, Takeya and Hanafusa point out homology between NA sequences common to both PR- and SR-RSV and a tract at the 3' end of the RAV-O D sequence, a likely region for cross-over on the viral RNA (Fig. 2c). They also used a probe for NA to detect homology in a *c-src* genomic clone downstream of the *c-src* coding region. However, this latter region of homology is not apparently transcribed, nor is it adjacent to the downstream sequence that forms the 3' end of *v-src*. It is thus difficult to see how this sequence participates in the recombination postulated in Fig. 2c.

A more intriguing question is the origin and function of the insertion of E. Takeya and Hanafusa could not find, by hybridization, any evidence for E sequences in cell DNA up to 11 kilobases 5' of *c-src* and 9 kilobases 3' of the gene. This lack of linkage with *src* accords with the finding of Swanson *et al.* that, by hybridization, E can be detected in the genome of viruses containing the oncogenes *v-myc* (MC29) and *v-erb* (AEV), although not in the genomes of several viruses lacking oncogenes nor in the transforming virus PRCII. The frequent, but not invariable, occurrence of E in viruses with oncogenes suggests either that they had a common ancestor, now lost (this seems unlikely since RSV, MC29 and AEV were first found in different parts of the world), or that E plays some part in the capture or functioning of viral oncogenes. Two findings are relevant to the latter possibility. Takeya and Hanafusa, by hybridization, detect E transcription in chick muscle but not chick embryo fibroblast cultures. The significance of this is unclear, nor does it bear on the observation of Schwartz *et al.* that the E sequence can potentially form the largest and most stable RNA or DNA 'hairpin' structure in the RSV genome. This structure reminds these authors of the 72-base pair enhancer element of murine leukaemia virus and they make the testable speculation that E is an enhancer, active no matter what side of *v-src* it is located.

Finally, of what significance in *v-src* evolution is the difference in the 3' ends of the coding regions of *v-src* and *c-src*? We do not yet know, but Takeya and Hanafusa stress that this is the only region where the viral and cell oncogenes differ markedly. This observation may be significant in understanding the relationship between *src*

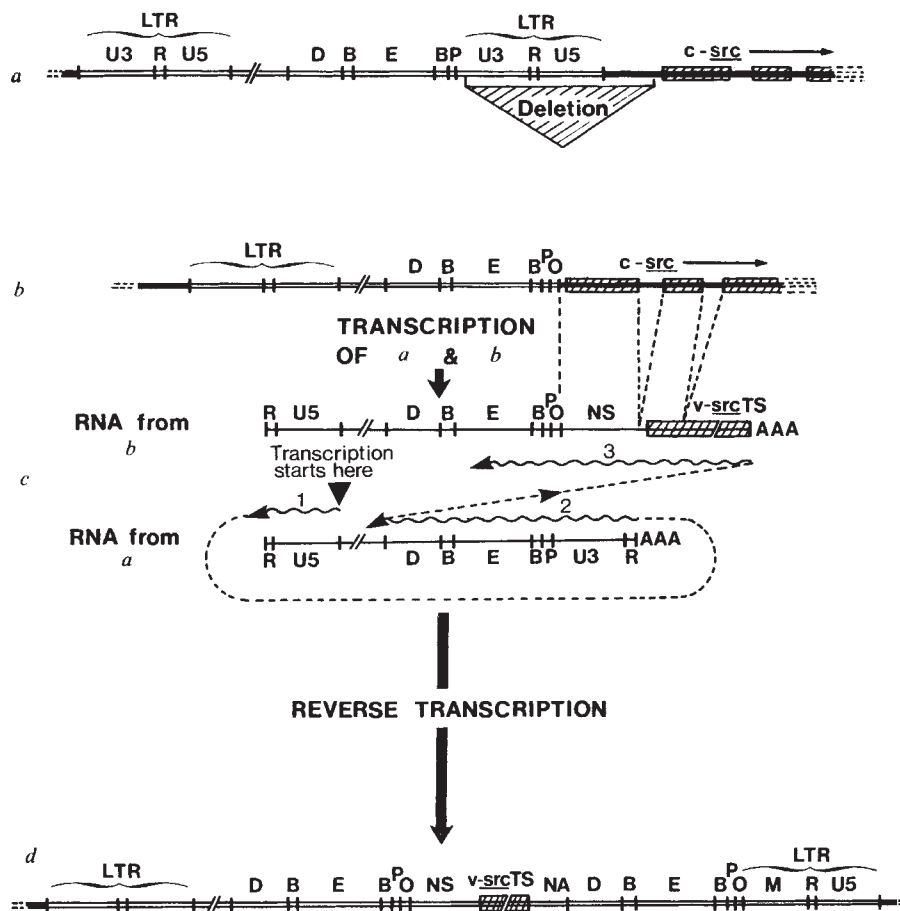


Fig. 2 Model for capture of *c-src* by a retrovirus. (Not to scale.) Single lines represent RNA; double lines, proviral DNA; heavy lines, cell DNA; wavy lines, DNA produced by reverse transcription, starting at the arrowhead in c and proceeding in the order 1, 2, 3 with a jump of templates between 1 and 2 and a cross-over between 2 and 3. Cross-hatched boxes: cell exons and viral coding regions. For explanation of lettering see Fig. 1 legend.

structure and function. The challenge here is to explain the pleiotropic effects of *v-src* activity in neoplasia, as well as the role of *c-src* in the normal cell, in terms of the functioning of the only known gene product, the 60,000-molecular weight phosphoprotein pp60<sup>src</sup>.

Virus mutants have proved useful in this extremely important task and Mardon and Varmus<sup>6</sup> have recently used mutants to demonstrate that matters might be even more complex than expected: pp60<sup>src</sup> may not be the only *src* gene product. Mardon and Varmus have exploited a transformed rat cell line, containing a single integrated provirus, in which morphological reversion and subsequent retransformation of the cell is accompanied by mutations in proviral *v-src*. In one such mutant, reversion seems to be due to a single-base-pair insertion causing a frameshift mutation and the synthesis of a nonfunctional truncated 18,000-molecular weight protein. Retransformation of this revertant occurred when a 242-base-pair duplication of sequences encompassing the deletion corrected the reading frame, producing a functional but enlarged 68,000-molecular weight protein.

However, of perhaps greater interest is the presence in both revertant and retrans-

formed cells of a 43,000-molecular weight protein whose translation begins in a different reading frame from that of pp60<sup>src</sup>, at an internal AUG just upstream of the mutation. As a result of the frameshift, most of this 43,000-molecular weight protein is the same as the carboxy terminus of pp60<sup>src</sup>, but in wild-type *v-src*, as well as in *c-src*, use of this internal AUG would produce a 7,000-molecular weight protein completely unrelated to pp60<sup>src</sup>. Since the mutant genes use this internal initiator codon it seems likely that it is also used by normal genes. The first task facing Mardon and Varmus is thus to demonstrate the existence of the 7,000-molecular weight *src* protein and, if it is found, they face the harder job of showing whether it contributes in any way to the function of the *src* gene. We cannot yet predict whether their efforts will complicate or clarify our ideas on the role of *src* in neoplasia. □

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1. Wang, J. Y. J. *Nature* **304**, (1983).
2. Waterfield, M. D. *et al.* *Nature* **304**, 35 (1983).
3. Schwartz, D. E., Tizard, R. & Gilbert, W. *Cell* **32**, 853 (1983).
4. Swanson, R., Parker, R., Varmus, H. E. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2519 (1983).
5. Takeya, T. & Hanafusa, H. *Cell* **32**, 881 (1983).
6. Mardon, G. & Varmus, H. *Cell* **32**, 871 (1983).



# Thrusting and duplex formation at Mount Isa, Queensland, Australia

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*Large-scale repetition of Mount Isa and Haslingden Group rocks in the anticline north of Mount Isa is interpreted as the core of a thrust duplex which has been subsequently folded parallel to the direction of thrusting. Displacement of Mount Isa Group rocks above the duplex is extremely significant to the regional geology and exploration for more Mount Isa style Pb–Zn and Cu orebodies. Interpretation of the geometry of the thrust belt indicates thrusting on different stratigraphical levels below and above the duplex during its formation, horses formed from a heelwards protruding footwall ridge instead of a trough, and the complex effects on stratigraphical cross-sections through duplexes of sloping lateral footwall ramps on horses.*

THE unusual repetition of Haslingden and Mount Isa Group rocks north of Mount Isa in Queensland, Australia (Fig. 1), has prompted several possible explanations: (1) block faulting and tilting associated with growth faults<sup>1</sup>; (2) spoon faulting in an extensional regime<sup>2</sup>; (3) large-scale arching of older rocks which were subsequently cut by east–west growth faults<sup>3</sup>.

These faults have been regarded by many<sup>1,3</sup> as the structural feeders for the mineralizing fluids which formed the enormous Mount Isa Pb–Zn and Cu deposits or the faults controlling possible repetition of the orebodies<sup>2</sup>. Detailed work by structural geologists at James Cook University and Mount Isa Mines has provided a framework which led to the following interpretation of the regional structural geology. The results of this fieldwork in the immediate Mount Isa vicinity are being prepared for publication. Here, only the rocks immediately to the north of Mount Isa itself are considered. However, this interpretation has been extended a considerable distance to the south of Mount Isa.

## Folding history

Three major macroscopic fold-producing deformations have affected the rocks shown in Fig. 1; these have been recently described for the area around Lake Moondarra<sup>4–6</sup>. The first deformation produced generally east–west oriented folds which are unusual in their distribution and localization because: (1) they generally only affect Mount Isa Group rocks and not the older Haslingden Group rocks (Fig. 1); (2) they are generally synclines; (3) they are usually located immediately to the south of major approximately east–west trending faults which uplifted (relatively) Haslingden Group rocks to the north (Fig. 1); and (4) their axial planes approximately follow the trend of these faults (Fig. 1). A weak slaty cleavage ( $S_1$ ) is sometimes developed parallel to their axial planes especially within the carbonaceous Breakaway Shale Formation.

The second deformation produced the macroscopic north–south oriented folds which dominate the regional 1:100,000 maps<sup>7–13</sup> and refolded  $D_1$  folds (compare with Crystal Creek Block in Fig. 1). A weak slaty-like cleavage ( $S_2$ ) is commonly associated with this deformation also. Only rarely has it been seen to crenulate  $S_1$  (refs 4, 6).

The third deformation formed NNW–SSE oriented folds and is only very locally developed (for example, at Mount Isa where it formed the Mount Isa Fold which affects the Pb–Zn orebodies<sup>14</sup>). It too locally develops a weak slaty-like cleavage ( $S_3$ ) which only very occasionally actually crenulates the earlier formed  $S_2$  cleavage<sup>4–6, 14</sup>.

## Faulting history

The only detailed recent structural work on fault timing in the Mount Isa Area is that of Winsor<sup>15</sup>, although other work is in progress (T.H.B. *et al.* in preparation). Well-documented and

consequently definite examples of growth faults are rare. The Moondarra Fault has well developed facies changes abutting it and structural timing criteria indicate that it is such a fault<sup>4, 15</sup>. Derrick<sup>3</sup> featured this and four other faults as examples of growth faults. Two of these, the Transmitter and Smith's faults, can be readily explained by younger faults with thickness changes resulting from proximity to the Mount Gordon Arch<sup>3</sup>. In particular, detailed structural work on the Transmitter Fault<sup>15</sup> indicates that it postdates  $D_3$ . The Investigator Fault is of considerable significance to the interpretation presented here and is discussed later as is the co-called 'Bonus Basin', which lies directly on the Mount Gordon Arch.

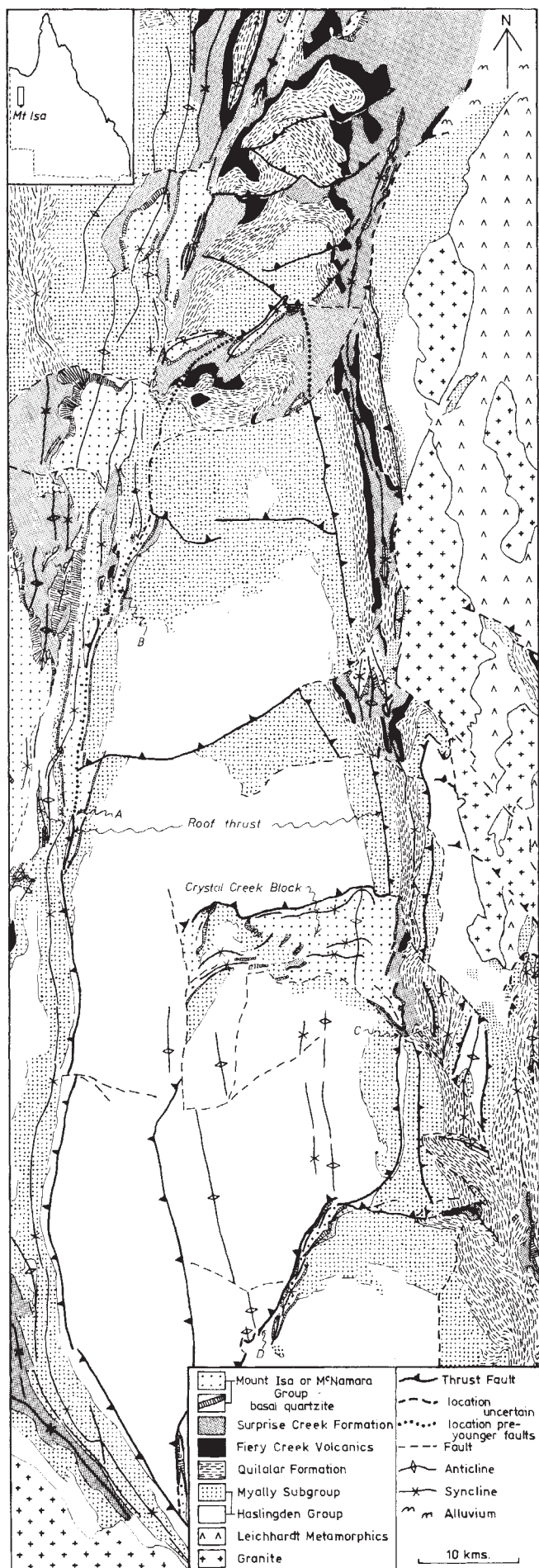
The faults shown in Fig. 1 can be interpreted in terms of Winsor's<sup>15</sup> timing relationships and our own detailed mapping to the south which documents a major east–west fault with a thrust geometry such that Eastern Creek Volcanics overlie Mount Isa Group rocks.

A tectonic/structural model of the area shown in Fig. 1 and that occurring farther south around Mount Isa, must explain first, the apparently complex distribution of fault blocks; second, the unusual geometry of large scale horizontal  $D_2$  synclines with a north–south trend lying to either side of a multiply east–west fault repeated anticline with a consistent moderate to steep plunge to the north; and third, the occurrence of approximately east–west trending  $D_1$  synclines in Mount Isa Group rocks immediately to the south of these east–west faults which do not appear to affect older Haslingden Group rocks or Mount Isa Group rocks within the horizontally plunging north–south synclines. These folds are also common in rocks to the south<sup>6</sup>.

## Interpretation

Figure 2 shows the geometry of horizontal synclines lying to either side of a steeply north plunging and fault repeated anticline. These structural relationships require either a fault between the anticline and its bounding synclines or a narrow zone of inhomogeneous strain across which the fold axes change progressively from horizontal to steeply plunging. No evidence for such a zone is shown on the 1:100,000 maps<sup>7–13</sup>. It is now considered to be a structurally-unlikely possibility as it would involve a totally brittle displacement on the east–west faults (with no evidence of a ductile shear zone) interacting with a totally ductile rotation on the approximately north–south trending boundaries to these blocks. It is more likely that both the east–west and north–south boundaries to these repeated blocks of north plunging anticline are faults. Dunnet<sup>2</sup> interpreted similar relationships and solved the spatial problems at depth (see Fig. 2) by regarding the east–west structures as listric faults which curved on to a decollement horizon.

A coherent, undisrupted fault does separate the horizontal western syncline from the north plunging anticline from half



**Fig. 1** Map showing the location of faults interpreted as thrusts and the stratigraphical relationships across them. The inset shows the location of the map within Queensland, Australia. A large-scale overall northerly plunging anticline runs down the centre of this map from north to south (this is also the approximate location of the Mt Gordon Arch). It is bounded to the east and west by essentially horizontally plunging synclines. The roof thrust and a significant lateral hanging wall ramp have been indicated (the latter occurs between C and D).

way down the map on the western side right through to Mount Isa itself. This is most readily apparent on Fig. 1 around location A where Haslingden Group rocks, from the limb of the horizontally plunging syncline to the west, are faulted against Mount Isa Group and Myally Subgroup rocks to the east. On the eastern side of the anticline this fault is not so apparent as it is to the west, because structurally it must occur within the Myally Subgroup. I have interpreted its location along the line separating approximately north-south striking beds to the east (due to their location on the horizontal syncline) from variably oriented beds which swing to the north-west (due to their involvement with the steeply north plunging anticline). This fault is, however, quite discernible in the Crystal Creek Block where Myally Subgroup structurally overlies Quilalar Formation (Fig. 1).

A critical structural relationship which leads to a solution of the timing of formation and origin of this fault block geometry is that the disposition of faulted anticlinal blocks relative to the adjacent synclines predates  $D_2$ . This has been demonstrated in north plunging fault repeats of the anticline to the south of the area shown in Fig. 1 (T.H.B. *et al.*, in preparation).  $L_2^2$ , the mineral elongation (stretching lineation) in  $S_2$  (terminology after ref. 16) pitches steeply south in  $S_2$  in both rotated anticlinal fault blocks as well as rocks with essentially horizontal fold axes to the west. Such a geometry could only have been produced as late as syn  $D_2$  if a zone of progressive rotation of  $F_2^0$  fold axes occurred between the synclines and anticline as described above. No such zone has been observed and, as discussed previously, it is structurally unlikely in this case.

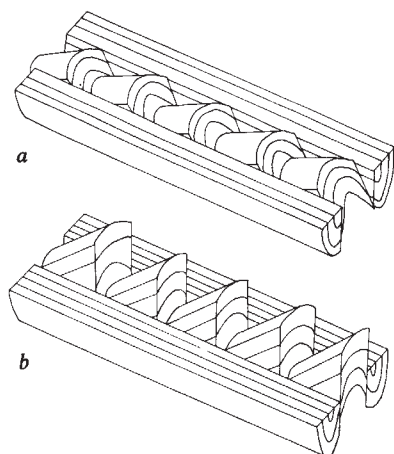
Consequently, it seems that before  $D_2$  the rocks within the anticlinal core were moderately to steeply north dipping except for some very local  $D_1$  folds, whereas those to either side were essentially horizontal except for some very local  $D_1$  folds.

An interpretation that explains this distinctive large scale geometry, and solves the problem of the east-west  $D_1$  synclines without associated anticlines in rocks immediately south of these major east-west trending faults, is that it results from a folded thrust duplex. The fault repeated northerly dipping stratigraphical succession within the anticlinal core forms a parcel of horses. These lie beneath the roof thrust which now consists of the horizontally plunging synclines to either side. Figure 3 shows the effect of folding a duplex and subsequently eroding the anticlinal core. Note the similarity between Fig. 3b and Fig. 1.

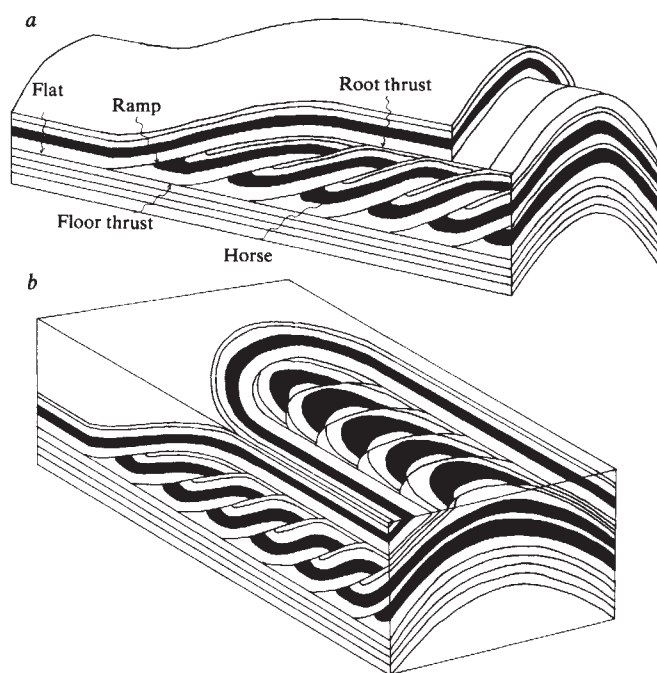
One difference of considerable significance is that the western syncline is faulted against progressively lower stratigraphical levels on each fault block. Such a geometry has been recorded in thrust duplexes<sup>17,18</sup> but no theoretical explanation has been advocated that fits currently accepted models of duplex development<sup>18-20</sup>. The geology of the Mount Isa area shown in Fig. 1 provides a ready explanation illustrated in Fig. 4 which shows a thrust sheet that has stepped up the stratigraphy across an irregularly shaped ramp. If the portion of the ramp protruding towards the heel of the thrust has outwards sloping sides and is subsequently imbricated, the roof of the duplex will ride over it and overlie all stratigraphical levels from the top to the bottom of the lateral footwall ramp.

The eastern exposure of the roof of the duplex does not give the theoretically expected geometry either. Thrusting occurred within the Myally Subgroup, yet the thrust lies along most of its length on the Myally Subgroup even though younger units are demonstrably being over-ridden in the core of the anticline (Fig. 1). How is this geometry possible when in a normal



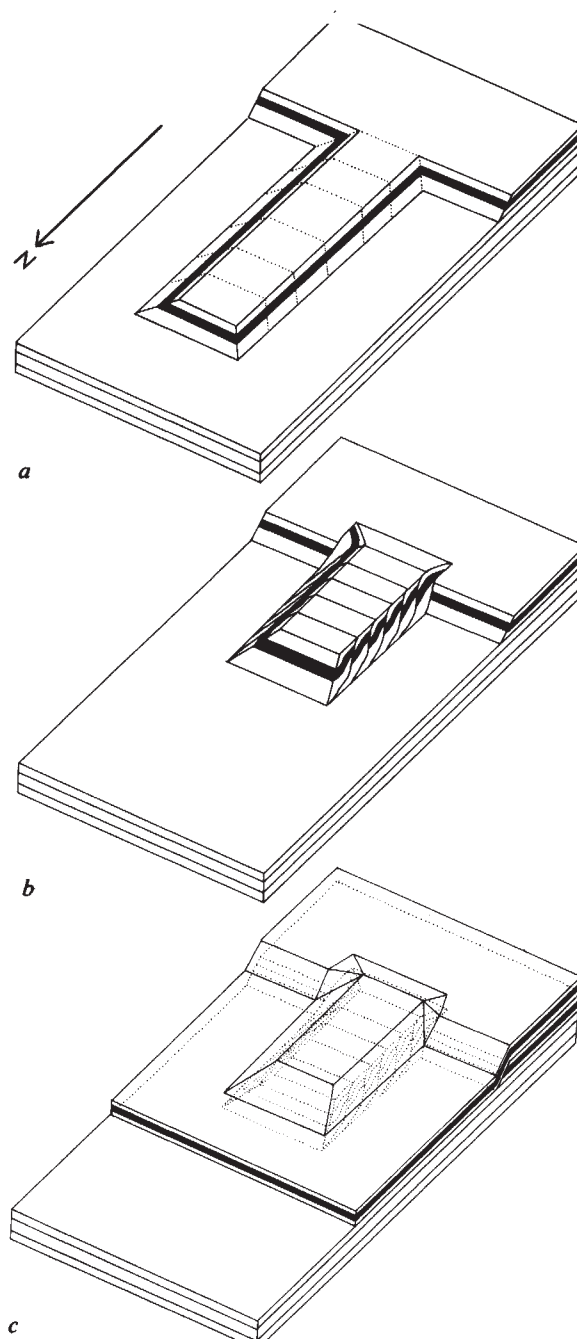


**Fig. 2** Previously accepted fault block relationships between the fault repeated anticlinal blocks and the bounding synclines. These sketches reveal a geometric problem generally not appreciated with this model. If the faults formed at right angles to bedding they must dip very shallowly to the south to accommodate plunges of  $60^\circ$  and  $60^\circ$  to the north of the anticlinal fold blocks whether or not they pre- or post-date folding (a). If the east-west fault repeats of the anticlinal core are steep, they must have originally dipped to the north before rotation of the bedding occurred to explain the steep fold axes plunges to the north (b).



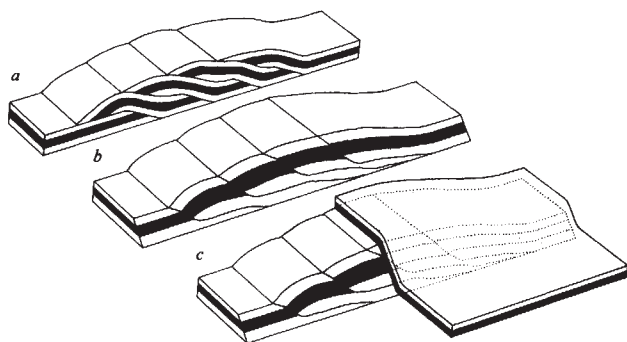
**Fig. 3** a, Currently accepted concepts of duplex geometry in cross-section, and the effect of folding this structure in the third dimension parallel to the movement direction on the thrusts that generated the duplex. Note that the lowest stratigraphical level in the roof thrust never comes in contact with similar stratigraphical levels in the horses except on the first formed (heelwards) ramp. b, Sketch showing the effects of eroding off the crest of the anticline on the exposure of the duplex shown in a. Note that internal geometric relationships of bedding and faults on the horizontal surface of this diagram are similar to the distribution shown in the map on Fig. 1. There are several important differences, however, particularly where the roof thrust comes in contact with the horses below.

cross-section one would expect to see the overlying thrust sheet cutting through to younger stratigraphical levels even if it was on a footwall lateral ramp (such as in Fig. 4b,c)? The significant factor is the angle of the footwall lateral ramp. If this ramp is around  $45^\circ$  then the larger width of the lower stratigraphical levels within the duplex means that they drape over the younger



**Fig. 4** A series of sketches showing the effect of imbricating an irregularity such as a heelward protruding lobe in a footwall ramp. a, The lobe has a  $45^\circ$  slope on the eastern lateral ramp and a  $70^\circ$  slope on the westward lateral ramp (incipient horse boundaries are dotted). b, The effect of imbricating this heel protruding lobe in the footwall ramp. c, The same structure as b with a roof thrust sheet in place. The geometrical relationships from the roof thrust to the western lateral footwall ramp are clear and quite significant. A single lowermost stratigraphical level within the roof thrust sheet moves from higher to lower stratigraphical levels within the horses down any individual horse at variance to generally accepted imbricated geometries as delineated in the cross-section in Fig. 3a. The eastern initially  $45^\circ$  sloping footwall lateral ramp is drawn more clearly from another view in Fig. 5.

higher levels, in subsequently formed (toewards) imbricate slices, as they ride up as shown in Fig. 5b. This means that the overlying thrust sheet can remain in contact, along the whole length of the duplex with one stratigraphical level halfway (for example) down the succession (Fig. 5c). This can subsequently be exposed due to later deformation and erosion, as in Fig. 1



**Fig. 5** Various geometric relationships within and on the footwall ramp of a duplex with a 45° slope on the lateral footwall ramp of the horses. *a*, A vertical cross-section through the horses parallel to the transport direction which has normal duplex geometry. *b*, The lateral footwall ramp of this structure which has a radically different geometry with little 'apparent' displacement due to the overlapping effects of the lower stratigraphical levels with their greater width. *c*, The overlying roof thrust which can be in contact with any stratigraphical level within the horses and in particular can remain in contact with a single stratigraphical level along any single approximately horizontal line along the lateral footwall ramp. These sketches were drawn from models constructed with plasticine and show accurately the effects of imbricating a lateral footwall with a 45° slope.

on the eastern edge of the anticline. Myally Subgroup at the base of the roof thrust remains in contact with Myally Subgroup in imbricates along the whole length of duplex. One exception to this is the Crystal Creek Block in which the roof thrust is in contact with Quilalar Formation (Fig. 1). This, however, only reinforces the above interpretation. The Crystal Creek Block is bounded against the Haslingden Group to the south by young faults which have caused an apparent oblique slip displacement of this block with both dextral and down dip components. The roof thrust would have been in contact with Quilalar Formation on the footwall lateral ramp below, higher up the western limb of the north-south syncline (see Fig. 5c).

The duplex seems to have formed by thrusting on higher stratigraphical levels for the roof thrust than the floor thrust. The level of thrusting lies within the Myally Subgroup around the macroscopic D<sub>2</sub> hinge and on the eastern limb of the anticline. On the western limb it steps down laterally (Fig. 1, location B), farther into the Haslingden Group (specifically to the basalts immediately below the Lena Quartzite). The floor thrust of the duplex seems to occur within or at the base of the Haslingden Group (specifically the Leander Quartzite) as these rocks are exposed within horses in the anticlinal core to the south (Fig. 1). Figure 6 demonstrates how thrusts can develop on two different stratigraphical levels across a duplex. Initially long flats and a single ramp form (Fig. 6a, compare with Myally Subgroup in Fig. 1). Subsequently thrusting initiates on a lower stratigraphical horizon possibly associated with towards propagation of a major decollement horizon. The duplex forms by imbrication off this floor thrust with continued displacement and roof thrust formation at the original higher stratigraphical level (Fig. 6b).

The floor thrust near the base of the Haslingden Group is probably joined farther to the west by the roof thrust, which must step laterally downwards immediately west of the western limb of the anticline because of the stratigraphical level of Haslingden Group exposed in the overthrust sheet in this region (Fig. 1).

## Discussion

Dunnet<sup>2</sup> recognized the geometric need for the zone of fault repeats of the anticlinal core to have bounding faults. However, he considered that these bounding faults were the Mount Gordon/Mount Isa and the Gorge Creek Faults. Locally, portions of the Mount Gordon and Mount Isa Faults, as in his Fig. 5,



**Fig. 6** The cross-section geometry (*b*) resulting from a change in stratigraphical level of thrusting from the floor to the roof thrust across the duplex due to initial thrusting at a higher stratigraphical level (*a*).

lie close to the location of the roof thrust as I have interpreted it. However, the Gorge Creek Fault is too far to the east. The recent 1:100,000 map sheets<sup>7-13</sup>, which reveal the extent of the horizontally plunging synclines well to the east of the Mount Gordon Fault, were not available by 1973 when Dunnet's work was finished. It is interesting that Dunnet's interpretation produces the type of listric normal fault distribution associated with extension at the heel of a major thrust belt<sup>21-23</sup>. Perkins<sup>24</sup> geometrically analysed Dunnet's interpretation, as applied to the Lake Moondarra area, in considerable detail from numerous holes drilled by Mount Isa Mines in that area, and concluded that it was geometrically untenable. Apart from this the greenstone basement was emplaced pre D<sub>2</sub> in the mine area (T.H.B. *et al.*, in preparation).

The role of growth-faults has been given considerable prominence in previous literature on the geology of Mount Isa, presumably because of the complex array of fault blocks and the considerable difficulty in explaining their distribution if one thinks purely in terms of up and down and sideways motion on faults. However, well documented examples of growth faults are rare and indeed their role may have been quite insignificant structurally in the tectonic development of the Mount Isa Block. Growth faults are not required for the development of the Mount Gordon Arch during deposition of the upper Haslingden and the Mount Isa/McNamara Groups. Many examples of the Warrina Park Quartzite cutting rapidly deeper into Haslingden Group rocks are shown on the Alsace<sup>8</sup>, Mary Kathleen<sup>9</sup> and Prospector<sup>10</sup> Sheets without any attendant faulting. The cause of the arch itself is presumably the emplacement of the Sybella Granite which occurred through to at least mid Mount Isa Group time<sup>3,26</sup> (see R. W. Page *et al.*, in preparation). Nevertheless, some growth faulting may have been associated with this arching.

The Investigator Fault was featured as a possible growth fault<sup>3</sup> because of thickness changes across it within the Gunpowder Creek Formation, combined with significant amounts of granule and pebble conglomerate on the southern side. This fault, however, is interpreted here as a thrust boundary of a horse within the duplex with a displacement across it of several kilometres (see Fig. 5a) and consequently the thickness and facies changes within the Gunpowder Creek Formation need no longer be so abrupt as to require the presence of a growth fault. The location of the Bonus Basin is intriguing and implies the presence of another subsidiary imbricate thrust slice which has not been shown on Fig. 1. However, no actual fault is exposed on its southern boundary and arching alone could account for its formation.

## Significance

This radically new interpretation of the regional structural geology has considerable significance to: regional geological and tectonic interpretations of the Mount Isa Province; exploration for economic mineralization of the Cu and Pb-Zn styles as seen at Mount Isa; and thrust belt development processes and geometry.

Current concepts of the regional geology and its tectonic development in terms of a Proterozoic Rift Zone have been summarized elsewhere<sup>3</sup>. However, although the Mount Gordon Arch was a very real tectonic feature controlling sedimentation, the apparent distribution of sediments to either side and across this arch is, in this thrust interpretation, dominated by the



geometry of imbricate slices within the duplex versus >234 km displacement of the roof. Consequently Mount Isa Group sediments from either side of the roof thrust were originally deposited hundreds of kilometres apart. However, since the tectonic structural controls on sedimentation were apparently also north-south oriented the effect is not quite as critical as if they had been east-west oriented.

The distribution of the Fiery Creek Volcanics, for example, is considerably simplified by the thrust interpretation. They occur on the roof thrust and those imbricates to the north end of the duplex, which explains their absence in the imbricate slices farther to the south yet their presence to the east and west.

The significance of this interpretation to exploration for Mount Isa style Pb-Zn and copper orebodies is fundamental, especially considering the origin of the copper<sup>14,24,25</sup> at Mount Isa relative to the structural history. This information cannot be discussed yet as it is waiting for company approval.

The geology of this region, if the thrust interpretation is correct, will enable us to develop an understanding of thrust

belt geometry and processes that is unparalleled. Here we have a large scale thrust belt in three dimensions due to the folding effects of the second deformation. At least 15 km of cross-section through this crust belt is exposed in the core of the anticlinal structure and this has already required previously undescribed thrust geometries to be predicted, modelled and applied. Ready explanations for cross-sectional relationships previously inexplicable in terms of accepted models of duplex development<sup>18-20</sup> have also been developed. The structural relationships also indicate that duplexes can form by thrusting on different stratigraphical levels above and below the package of horses. These relationships and the results of further work on this area are of considerable interest, especially in the light of recent COCORP<sup>27,28</sup> revelations on the significance of deep levels of thrusting in the tectonic development of various regions in the USA such as the Appalachians.

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1. Smith, W. D. *Spec. Publ. Geol. Soc. Aust.* **3**, 225-235 (1969).
2. Dunnet, D. *Phil. Trans. R. Soc. A* **283**, 333-344 (1976).
3. Derrick, G. M. *BMR J. Aust. Geol. Geophys.* **7**, 81-92 (1982).
4. Winsor, C. N. thesis, James Cook Univ. (1982).
5. Winsor, C. N. *Tectonophysics* **92**, 195-210 (1983).
6. Winsor, C. N. *J. geol. Soc. Aust.* (submitted).
7. Hutton, I. et al. *BMR Aust. Prelim. Map Sheet* 6758 (1982).
8. Derrick, G. M. & Wilson, I. H. *BMR Aust. Rec.* 1982/6 (1982).
9. Derrick, G. M., Wilson, I. H., Hill, R. M., Glikson, A. Y., Mitchell, J. E. *BMR Aust. Bull.* 193 (1977).
10. Wilson, I. H. et al. *BMR Aust. Rec.* 1977/4 (1977).
11. Wilson, I. H., Hill, R. M., Noon, T. A., Duff, B. A. & Derrick, G. M. *BMR Aust. Rec.* 1979/24 (1979).
12. Wilson, I. H. et al. *BMR Aust. Prelim. Map Sheet* 6859 (1981).
13. Derrick, G. M. et al. *BMR Aust. Prelim. Map Sheet* 6759/6760 (1980).
14. Swager, C. P. thesis, James Cook Univ. (1983).
15. Winsor, C. N. *J. struct. Geol.* (submitted).
16. Bell, T. H. & Duncan, A. C. *Tectonophysics* **47**, T1-T5 (1978).
17. Willis, B. *Bull. geol. Soc. Am.* **13**, 305-352 (1902).
18. Boyer, S. E. & Elliott, D. *Am. Ass. petrol. Geol.* **66**, 1196-1230 (1982).
19. Elliott, D. & Johnson, M. R. *W. Trans. R. Soc. Edinb.* **71**, 69-96 (1980).
20. Butler, R. W. H. *J. struct. Geol.* **4**, 239-245 (1982).
21. Dahlstrom, C. D. A. *Bull. Can. petrol. Geol.* **18**, 332-406 (1970).
22. Bally, A. W. *Phil. Trans. R. Soc. A* **305**, 325-338 (1982).
23. Bally, A. W. *Oceanol. Acta, Proc. 26th int. Congr.* 87-101 (1981).
24. Perkins, W. G. *Mt Isa Mines Ltd. Int. Rep.* 120/2 (1979).
25. Perkins, W. G. *Econ. Geol.* (submitted).
26. Page, R. W. *J. geol. Soc. Aust.* **25**, 141-164 (1978).
27. Cook, F. A. et al. *Geology* **7**, 563-567 (1979).
28. Cook, F. A. & Oliver, J. E. *Am. J. Sci.* **281**, 993-1008 (1981).

# Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1

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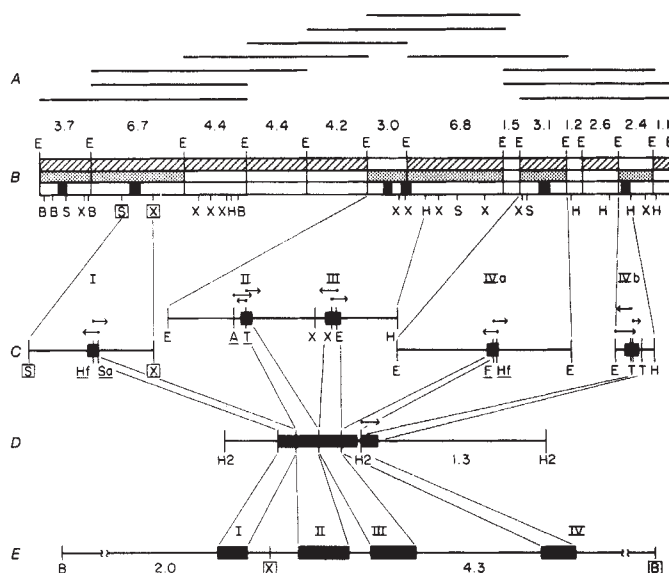
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*The homologue of the viral Kirsten ras (v-Ki-ras) gene found in the human lung carcinoma cell line, Calu-1, has an intron-exon structure similar to that of the human homologue of the viral Harvey ras (v-Ha-ras) gene. A second, potential fourth coding exon is present in the human Ki-ras gene and similar sequences are found in the Kirsten murine sarcoma virus. Cysteine is encoded at the twelfth amino acid position, suggesting that the Calu-1 Ki-ras gene has undergone a mutational activation at the same position as the human Ha-ras gene of the bladder carcinoma cell line, T24. A comparison of their predicted amino acid sequences suggests that ras proteins have a 'constant' region and a 'variable' region. Here we propose a common modular structure for ras gene products in which the variable region forms a physiologically important combining site.*

THE *ras* genes were first characterized as the transforming genes of the Harvey and Kirsten murine sarcoma viruses (v-Ha-ras and v-Ki-ras, respectively)<sup>1</sup>. These viral *ras* genes share considerable nucleotide homology and encode structurally

related 21,000-molecular weight proteins (p21)<sup>1</sup>. Each arose by transduction of a distinct cellular rat gene, and is highly conserved in evolution<sup>1</sup>. Use of DNA-mediated gene transfer has shown that DNAs from many human tumours and human tumour cell lines contain *ras* genes capable of tumorigenic and morphological transformation of NIH 3T3 murine fibroblasts<sup>2-5</sup>. The transforming gene of T24 bladder carcinoma cells is the human homologue of the viral Ha-ras<sup>2-4</sup>. A transforming gene commonly found in human carcinomas of lung, colon and

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**Fig. 1** A, Representative overlapping phage inserts from Charon 4A libraries containing portions of the human Ki-ras gene cloned from NIH 3T3 cells transformed with Calu-1 DNA. The restriction map of the Ki-ras gene is shown in B. Hatched regions indicate *EcoRI* fragments having homology to human repeat sequences as determined by strong hybridization with  $^{32}\text{P}$ -labelled nick-translated human DNA<sup>21</sup>. Stippled regions indicate *EcoRI* fragments having homology to the pKBE-2 clone of Ki-MuSV<sup>1</sup>. Solid regions indicate more precisely the region of viral Ki-ras homology. C, An expansion of the regions of viral Ki-ras homology showing the strategy used in nucleotide sequence determination. Arrows indicate regions sequenced by the method of Maxam and Gilbert<sup>22</sup> after 3' OH labelling<sup>23</sup>. D, Map of the viral Ki-ras gene. The regions of homology between the human and viral genes are indicated, as is the strategy used to sequence the alternate IVb coding exon of the viral Ki-ras. E Shows the structure of the human Ha-ras gene, as determined previously<sup>11,12</sup>. Solid boxes indicate coding regions. Regions of homology between the human Ha-ras and viral Ki-ras are indicated. Numbers indicate the distance in kbp. Restriction endonuclease sites are: E, *EcoRI*; B, *BamHI*; S, *SmaI*; X, *XbaI*; H, *HindIII*; Hf, *HinfI*; Sa, *Sau3A*; A, *AhaIII*; T, *TaqI*; F, *FokI*; H2, *HincII*. Restriction sites that are underlined are not unique for the restriction fragment shown. Restriction sites in boxes frame DNA fragments that were used in the chimaeric gene construction described in the text.

other tissues is the homologue of the viral Ki-ras<sup>2,5</sup>. The transforming gene of the SK-N-SH neuroblastoma cell line is called N-ras; it encodes a protein structurally related to the other ras gene products<sup>5</sup>. These transforming genes presumably arise by a somatic mutational 'activation' of the respective cellular ras genes<sup>6-8</sup>.

## Cloning the Ki-ras gene

We have cloned the transforming Ki-ras gene of Calu-1, a human lung carcinoma cell line<sup>9</sup>. Primary transformants of NIH 3T3 cells were generated by gene transfer with Calu-1 DNA. DNA from primary transformants was then used to generate secondary and, in turn, tertiary, NIH 3T3 transformants. Phage libraries were constructed in Charon 4A from secondary and tertiary transformant DNAs which had been partially cleaved with the restriction endonuclease *EcoRI*. The first Ki-ras phage clone was obtained by screening these libraries with the human repeat probe, BLUR8, as briefly described elsewhere<sup>5</sup>. This clone,  $\lambda$ L2-34, was cleaved with restriction endonucleases, and restriction fragments of phage insert were used for further screening of phage libraries constructed from DNA of NIH 3T3

transformants. This process was reiterated. In this manner we obtained 34 phage with overlapping restriction maps spanning 45 kilobase pairs (kbp). This entire region was mapped for *XbaI*, *SmaI*, *BamHI*, *HindIII* and *EcoRI* restriction endonuclease cleavage sites, and resulting DNA fragments were blotted and screened for homology to human repeat sequence and viral Ki-ras sequences. The results are shown in Fig. 1. We obtained all the *EcoRI* fragments containing human repeat sequences which are reproducibly associated with this gene on gene transfer (in kbp): 6.8, 6.7, 4.4, 4.2, 3.7, 3.1, 2.6 and 1.1 (ref. 9). Similarly, we obtained all *EcoRI* fragments having homology to a plasmid clone of viral Ki-ras, pKBE-2 (ref. 1), which are reproducibly associated with this gene (in kbp): 6.8, 6.7, 3.1, 3.0 and 2.4 (ref. 5). One novel *EcoRI* fragment of 3.7 kbp, which had not been previously appreciated from genomic blots with viral Ki-ras probes, had homology to sequences 5' to viral Ki-ras coding sequences (data not shown). Finally, the 2.4-kbp *EcoRI* fragment hybridized to a region of the Kirsten murine sarcoma virus (Ki-MuSV) 3' to the canonical ras coding sequences. We conclude that we have cloned an intact human Ki-ras gene.

## Ki-ras coding regions

We prepared a finer restriction endonuclease map of all regions having homology to the viral Ki-ras gene. These regions were sequenced as described in Fig. 1 legend. Comparison of the predicted amino acid sequence of the Ki-ras with that of the published viral Ki-ras<sup>10</sup> allowed an unambiguous assignment of the coding exons. Four coding exons (labelled I, II, III and IVa in Fig. 1) together would comprise an 189-amino acid protein product differing from the viral Ki-ras p21 at only seven positions (see Fig. 2). Consensus splice sequences were found at each intron-exon boundary (data not shown). The placement of these boundaries relative to the encoded protein corresponded exactly to the intron-exon boundaries of the human Ha-ras gene<sup>11,12</sup> (see Fig. 2), consistent with the notion that Ha-ras and Ki-ras both evolved from a common, similarly spliced ancestral gene.

In addition to the first four coding exons, we found a potential coding exon (labelled IVb in Fig. 1) having homology to a region 3' to the termination codon of the viral Ki-ras gene. Both the human and viral regions were sequenced. The Ki-ras IVb sequence is preceded by a consensus splice sequence, and consists of an open reading frame which would translate into 38 amino acids, one less than would be found in Ki-ras IVa. The Ki-ras IVa- and IVb-encoded amino acid sequences are also quite similar, encoding a related 14-amino acid sequence at the 5' end of the exon and a related 5-amino acid sequence at the 3' end (see Fig. 3). The intervening amino acids (20 for Ki-ras IVa and 19 for Ki-ras IVb) are highly divergent. These two exons (IVa and IVb) have diverged from each other in nearly the same manner as the fourth exons of N-ras, Ha-ras and Ki-ras have each diverged from each other (see Fig. 3). Analysis of cDNA clones from human Ki-ras transformants indicates that both the IVa and IVb exonic sequences are expressed (M.G., unpublished results). Precedent exists for multiple gene products due to differential splicing of transcripts from the same genetic locus in vertebrates<sup>13-15</sup>.

Examination of the viral Ki-ras IVb region indicates that sequences similar to the human Ki-ras IVb must exist in the rat genome. Viral Ki-ras IVb sequences begin 5 base pairs (bp) 3' to the Ki-ras termination codon. The human and viral IVb sequences encode a similar amino acid sequence up to and including a poly-lysine stretch (Fig. 3). After this, there appears to be complete divergence of amino acid sequence. However, this part of the viral Ki-ras IVb can also be aligned with the human Ki-ras IVb by a 5-bp deletion within the oligo(dA) stretch (Fig. 3), yielding again a strikingly similar amino acid sequence. We propose, therefore, that a Ki-ras IVb coding



Calu-1 K-ras ATG ACT GAA TAT AAA CTT GTG GTA GTT GGA GCT TGT GGC GTA GGC AAG AGC GGC TTG ACG  
 viral K-ras ATG ACT GAA TAT AAA CTT GTG GTA GTT GGA GCT TGT GGC GTA GGC AAG AGC GGC TTG ACG  
 Calu-1 K-ras met thr glu tyr lys leu val val val gly ala asp gly val gly lys ser ala leu thr

Calu-1 K-ras ATA CAG CTA ATT CAG AAT CAT TTT GTG GAC GGA TAT GAT CCA AGA ATA GAG GAT TCC TAC  
 viral K-ras ATA CAG CTA ATT CAG AAT CAT TTT GTG GAC GGA TAT GAT CCA AGA ATA GAG GAT TCC TAC  
 viral K-ras ile gln leu ile gln asn his phe val asp gly tyr asp pro thr ile asp asp ser tyr  
 Calu-1 K-ras ile gln leu ile gln asn his phe val asp gly tyr asp pro thr ile asp asp ser tyr

Calu-1 K-ras AGG AAG CAA GTA GTA ATT GAT GGA GAA ACC TOT CTC TTG GAT ATT CTC GAC ACA GCA GGT  
 viral K-ras AGG AAG CAA GTA GTA ATT GAT GGA GAA ACC TOT CTC TTG GAT ATT CTC GAC ACA GCA GGT  
 viral K-ras arg lys gln val val file asp gly glu thr oys leu leu asp ile leu asp thr ala gly  
 Calu-1 K-ras arg lys gln val val file asp gly glu thr oys leu leu asp ile leu asp thr ala gly

Calu-1 K-ras CAA GAG GAG TAC AGT GCA ATG AGG GAC CAG TAC ATG AGG ACT GGG GAG GGC TTT CTT TGT  
 viral K-ras CAA GAG GAG TAC AGT GCA ATG AGG GAC CAG TAC ATG AGG ACT GGG GAG GGC TTT CTT TGT  
 viral K-ras gln glu glu tyr ser ala met arg asp gln tyr met arg thr gly glu gly phe leu oys  
 Calu-1 K-ras gln glu glu tyr ser ala met arg asp gln tyr met arg thr gly glu gly phe leu oys

Calu-1 K-ras GTA TTT GGC ATA AAT AAT ACT AAA TCA TTT GAA GAT ATT CAC CAT TAT AGA GAA CAA ATT  
 viral K-ras GTA TTT GGC ATA AAT AAT ACT AAA TCA TTT GAA GAT ATT CAC CAT TAT AGA GAA CAA ATT  
 viral K-ras val phe ala ile asn asn thr lys ser phe glu asp ile his his tyr arg glu gln leu  
 Calu-1 K-ras val phe ala ile asn asn thr lys ser phe glu asp ile his his tyr arg glu gln leu

Calu-1 K-ras AAA AGA GTT AAG GAC TCT CAA GAT GAT CCT ATG GTC CTA GTA GGA AAT AAT TGT GAT TTG  
 viral K-ras AAA AGA GTT AAG GAC TCT CAA GAT GAT CCT ATG GTC CTA GTA GGA AAT AAT TGT GAT TTG  
 viral K-ras lys arg val lys asp ser glu asp val pro met val leu val gly asn lys oys asp leu  
 Calu-1 K-ras lys arg val lys asp ser glu asp val pro met val leu val gly asn lys oys asp leu

Calu-1 K-ras CCT TCT AGA ACA GTA GAC AGA AAA CAG GCT CAG GAC TTA GCA AGA AGT TAT GAT ATT CTT  
 viral K-ras CCT TCT AGA ACA GTA GAC AGA AAA CAG GCT CAG GAC TTA GCA AGA AGT TAT GAT ATT CTT  
 viral K-ras pro ser arg thr val asp thr lys gln ala gln glu leu ala arg ser tyr gly ile pro  
 Calu-1 K-ras pro ser arg thr val asp thr lys gln ala gln asp leu ala arg ser tyr gly ile pro

Calu-1 K-ras TTT ATT GAT GAT TCA GCA AGA AAG ACA AGA CAG AGA GTC GAG GAT GCT TTT TAT ACA TTG GTC  
 viral K-ras TTT ATT GAT GAT TCA GCA AGA AAG ACA AGA CAG AGA GTC GAG GAT GCT TTT TAT ACA TTG GTC  
 viral K-ras phe ile glu thr thr ser ala lys thr arg gln arg val glu asp ala phe tyr thr leu val  
 Calu-1 K-ras phe ile glu thr thr ser ala lys thr arg gln arg val glu asp ala phe tyr thr leu val

Calu-1 K-ras AGA GAG ATC CGA CAA TAC AGA TTT AAA AAA ATC AGC AAA GAA GAA AAG ACT COT GGC TOT  
 viral K-ras AGA GAG ATC CGA CAA TAC AGA TTT AAA AAA ATC AGC AAA GAA GAA AAG ACT COT GGC TOT  
 viral K-ras arg glu ile arg gln tyr arg leu lys lys ile ser lys glu glu lys thr pro gly oys  
 Calu-1 K-ras arg glu ile arg gln tyr arg leu lys lys ile ser lys glu glu lys thr pro gly oys

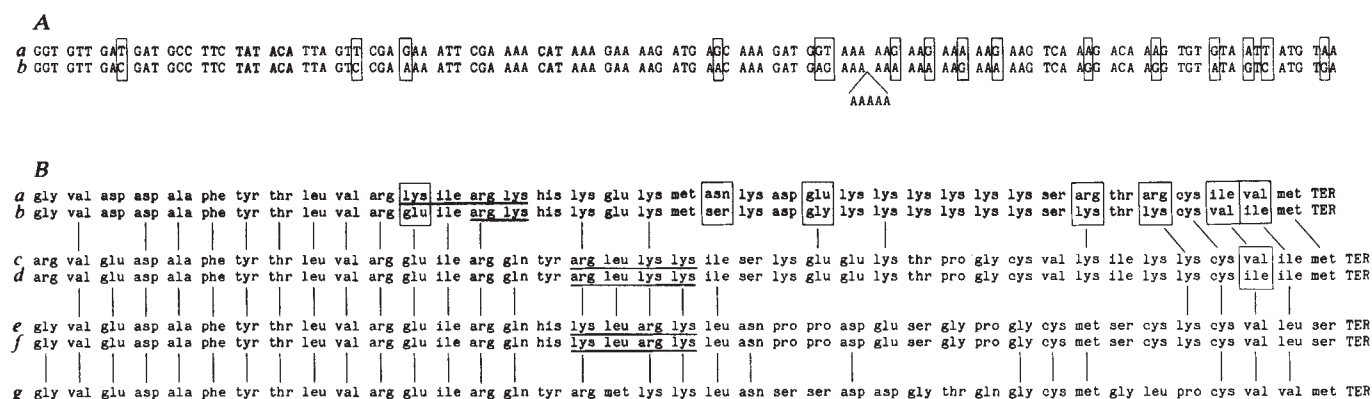
Calu-1 K-ras GTT AAA ATT AAA AAA TGC ATT ATA ATG TAA  
 viral K-ras GTT AAA ATT AAA AAA TGC ATT ATA ATG TAA  
 viral K-ras val lys ile lys lys oys val ile met TER  
 Calu-1 K-ras val lys ile lys lys oys val ile met TER

**Fig. 2** The complete nucleotide sequence and predicted amino acid sequence of the human *Ki-ras* gene (exons I, II, III and IVa) are shown together with the corresponding region of the viral *Ki-ras* gene determined by others<sup>10</sup>. Sequence differences are boxed. Arrowheads mark the splice sites of the human *Ki-ras* gene.

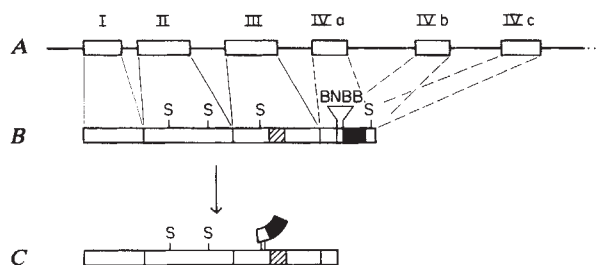
exon exists in rat with an amino acid sequence close to that shown in Fig. 3. We further propose that this sequence was transduced during the biogenesis of Ki-MuSV and, at some time during viral propagation or molecular cloning, an error in replication occurred, perhaps by polymerase slippage, in the dA-rich region which encodes a poly-lysine stretch. Since there has probably been no functional selection for the viral *Ki-ras* IVb sequence, it may also have diverged from the normal rat cellular sequence in other respects.

## Transforming potential of Calu-1 *Ki-ras* gene

The *Ki-ras* gene of Calu-1, which transforms NIH 3T3 cells, differs from the *Ki-ras* gene of normal human DNA, which does not<sup>9</sup>. To determine the basis for this difference, we cloned a normal *Ki-ras* gene by screening a Charon 4A library constructed from human placental DNA. We obtained all but the 5'-most 3.7-kbp *EcoRI* fragment. The *EcoRI* restriction endonuclease map for the remaining 41.4 kbp was identical to that of the Calu-1 *Ki-ras* gene. It is possible that the difference between the normal and transforming *Ki-ras* genes results from small nucleotide changes. Indeed, the transforming potential of the normal *Ha-ras* gene can be activated by any mutation substituting valine<sup>6-8</sup>, arginine, aspartic acid or serine for glycine at amino acid position 12 (O.F., work in progress). Sequence comparisons of the first coding exons from the Calu-1 and normal *Ki-ras* gene showed two nucleotide differences. Significantly, Calu-1 *Ki-ras* encodes cysteine (TGT) at position 12 while the normal *Ki-ras* encodes glycine (GGT). Also, Calu-1 *Ki-ras* encodes glycine (GGA) at position 31 while the normal *Ki-ras* encodes glutamic acid (GAA) at that position. To test whether these sequence differences could account for the transforming capacity of the Calu-1 *Ki-ras* gene, the first coding exons of the Calu-1 and the normal *Ki-ras* genes were each joined by DNA ligation to the last three coding exons of a normal human *Ha-ras* gene. This construction used an *XbaI* site in the intron that separates the first from the second coding exons in both *Ha-ras* and *Ki-ras* genes (see Fig. 1). Only the chimaeric gene containing the first coding exon from Calu-1 *Ki-ras* was able to induce morphologically transformed foci of NIH 3T3 cells.



**Fig. 3** A Shows the complete nucleotide sequence of the human *Ki-ras* IVb exon (a) and its viral equivalent (b). Boxes indicate sequence differences. B, The predicted amino acid sequences of: a, rat *Ki-ras* exon IVb inferred from the Kirsten viral sequence<sup>10</sup>; b, human *Ki-ras* exon IVb; c, rat *Ki-ras* exon IVa inferred from the Kirsten viral sequence<sup>10</sup>; d, human *Ha-ras* exon IVa; e, rat *Ha-ras* exon IV inferred from the Harvey viral sequence<sup>24</sup>; f, human *Ha-ras* exon IV<sup>11,12</sup>; g, human *N-ras* exon IV (E.T., unpublished results). Differences between the human and rat homologues are boxed. Many of these differences are conservative (Arg for Lys or Ile for Val or Leu). Positions where the different members of the *ras* family contain the same or closely related amino acids are indicated by connecting lines. The sequence BNBB, described in the text—a possible proteolytic cleavage sequence—is underlined.



**Fig. 4** Hypothetical structures of the *ras* genes and their products. **A**, The exonic arrangement of the *ras* genes with three coding exons (I, II and III) and alternate fourth coding exons (IVa, IVb, IVc and so on). The gene encodes a product, p21, 188–189 amino acids long (**B**) with domains encoded by the exons as indicated. The primary gene products have one highly variable region, indicated in black, and one less variable region, indicated by cross-hatching, which distinguish the individual members of the *ras* gene family but are conserved between species (rat and man). Preceding the highly variable region is the sequence BNBB (basic amino acid, any amino acid, basic, basic amino acid) which we postulate to be a proteolytic cleavage site. We suggest that in its mature form the terminal peptide containing the variable region is connected to the main body of the p21 through a disulphide linkage (||) to one of the cysteine residues indicated (S). Disulphide linkage of the type shown in **C** would bring the two variable regions into apposition, creating a physiologically important combining site unique for each member of the *ras* family.

## Structure of *ras* genes and their products

A clearer picture is emerging of the relatedness of the *ras* gene products. First, there is strong conservation of the entire amino acid sequence between homologous *ras* genes in distant species. Thus, the human Ha-*ras* gene differs from the viral Ha-*ras* gene, derived from rat, at only 3 out of 189 amino acid positions, despite a high frequency of third-base neutral changes<sup>11,12</sup>. Similarly, the human Ki-*ras* gene (comprised of exons I, II, III and IVa) differs from the viral Ki-*ras* gene, also derived from rat, at only 7 of 189 positions. We believe this conservation reflects evolutionary pressure on the maintenance of the physiological role of the *ras* gene products.

Second, the individual members of the *ras* gene family show a high degree of relatedness at the amino acid sequence level for amino acids 1–120, 133–163 and 185–189. In this 'constant' region the human Ki-*ras* and Ha-*ras* differ at only seven positions. The human N-*ras* gene shows a similar conservation in this region (E.T., unpublished results). However, the predicted amino acid sequence markedly diverges at amino acids 164–184, and less so at positions 121–132. We call these 'variable' regions (see Fig. 4). The sequence in the variable regions is, however, conserved between species. Thus there are no amino acid changes between the human and viral Ha-*ras* genes in this region and few significant differences between the human and viral Ki-*ras* genes. This region may, therefore, determine the physiological role of the individual *ras* gene products.

We wish to speculate further about the common structure of the *ras* gene products. In each case, the region of divergent amino acid sequence (171–184) begins with the amino acid sequence BNBB where B is either lysine or arginine and N is any amino acid. The dibasic amino acid sequence is a common recognition sequence for proteolytic cleavage of many membrane-associated and secreted proteins. The particular sequence BNBB is also commonly found at proteolytic cleavage sites, such as in preproinsulin<sup>16,17</sup> and viral glycoprotein precursors<sup>18</sup>. Moreover, it is known from the work of others that the *ras* p21s undergo a carboxy-end proteolytic cleavage during maturation<sup>19</sup>. By comparing the electrophoretic mobility of synthesized, mature Ha-*ras* p21 with *in vitro*-translated Ha-*ras* p21 on reducing SDS-polyacrylamide gels, we estimate that the cleavage site is ~20 amino acids from the carboxy end. This would place cleavage quite near the BNBB sequence, separating the constant region from the variable region encoded by exon IV. What then is the fate of this variable region? One possibility is that after cleavage the terminal peptide serves as a hormone, growth factor or second messenger. Another possibility, the one we favour, is that this terminal peptide is retained by the *ras* p21 complex. Indeed, the last five amino acids of *ras* p21s are conserved and contain a cysteine residue, raising the possibility of a disulphide linkage of the terminal variable region with the main body of *ras* p21s. There are three conserved cysteine residues in the main body of the *ras* p21. One of these, encoded in the third exon, is immediately adjacent to another, less extensive, variable region. If disulphide linkage occurred here, a single, joint variable region would result (see Fig. 4). We propose that this region might serve as a combining site, allowing each different *ras* gene product to interact with a different cellular or extracellular constituent.

Finally, we wish to speculate about the structure of the *ras* genes themselves. The exonic structures of the human Ha-*ras* and Ki-*ras* genes are quite similar, indicating that both have evolved from a similarly spliced ancestral gene. However, an alternative fourth coding exon was found in the Ki-*ras* gene by virtue of the presence of homologous sequences in the Ki-MuSV genome. Further alternate fourth coding exons may be present, not only in the Ki-*ras* gene but in the other *ras* genes also. These might not be readily detected by hybridization analysis if they are as diverged as Ki-*ras* IVa is from Ki-*ras* IVb. Thus, not only are there at least five members of the *ras* gene family<sup>3,20</sup>, but each member may be able to express more than one physiologically distinct protein. The control of the expression of these genes and their gene products is probably prominent in growth control, development and associated pathology.

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1. Ellis, R. *et al.* *Nature* **292**, 506–511 (1981).
2. Der, C., Krontiris, T. & Cooper, G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3637–3640 (1982).
3. Parada, L., Tabin, C., Shih, C. & Weinberg, R. *Nature* **297**, 474–478 (1982).
4. Santos, E., Tronick, S., Aaronson, S., Pulciani, S. & Barbacid, M. *Nature* **298**, 343–347 (1982).
5. Shimizu, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **80**, 2112–2116 (1983).
6. Taparowsky, E. *et al.* *Nature* **300**, 762–765 (1982).
7. Reddy, E., Reynolds, R., Santos, E. & Barbacid, M. *Nature* **300**, 149–152 (1982).
8. Tabin, C. *et al.* *Nature* **300**, 143–148 (1982).
9. Peruchio, M. *et al.* *Cell* **27**, 467–476 (1981).
10. Tsuchida, N., Ryder, T. & Ohtsubo, E. *Science* **217**, 937–939 (1982).
11. Fasano, O., Taparowsky, E., Fiddes, J., Wigler, M. & Goldfarb, M. *J. molec. appl. Genet.* (in the press).

12. Capon, D., Chen, E., Levinson, A., Seeburg, P. & Goeddel, D. *Nature* **302**, 33–37 (1983).
13. Ait, F. *et al.* *Cell* **20**, 293–301 (1980).
14. Early, P. *et al.* *Cell* **20**, 313–319 (1980).
15. Amara, S., Jonas, V., Rosenfeld, M., Ong, E. & Evans, R. *Nature* **298**, 240–244 (1982).
16. Ullrich, A. *et al.* *Science* **196**, 1313–1319 (1977).
17. Perler, F. *et al.* *Cell* **20**, 555–566 (1980).
18. Rice, C. & Strauss, J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2062–2066 (1981).
19. Shih, T. *et al.* *J. Virol.* **42**, 253–261 (1982).
20. Chang, E., Gonda, M., Ellis, R., Scolnick, E. & Lowy, D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4848–4852 (1982).
21. Maniatis, T., Jeffrey, A. & Kleid, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1184–1188 (1975).
22. Maxam, A. & Gilbert, W. *Meth. Enzym.* **65**, 499–580 (1980).
23. Drouin, J. *J. molec. Biol.* **140**, 15–34 (1980).
24. Dahr, R. *et al.* *Science* **217**, 934–937 (1982).



# Structure and organization of the human *Ki-ras* proto-oncogene and a related processed pseudogene

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*Analysis of the organization and nucleotide sequence of two human loci related to the transforming gene of Kirsten murine sarcoma virus establishes one as a functional gene and the other as a processed pseudogene. The two final coding exons of the functional gene seem to have arisen by duplication. Differentially spliced mRNAs incorporating one or other of the duplicated exons probably served as the intermediates by which the viral transforming gene and the pseudogene were generated. This suggests that the functional gene may specify either of two related polypeptides depending on the pattern of RNA splicing.*

THE use of DNA-mediated gene transfer techniques has revealed the existence of activated cellular oncogenes in several human tumour cell lines and solid tumours (for review, see ref. 1). Such genes, which efficiently induce the transformation of NIH 3T3 cells, have been identified in human bladder, colon, lung and mammary carcinoma cell lines<sup>2-4</sup>, promyelocytic leukaemia<sup>5</sup>, neuroblastoma<sup>5</sup> and sarcoma cell lines<sup>6</sup>, and unmanipulated solid tumours including carcinomas of the lung, colon and pancreas<sup>7</sup>. Recent work has demonstrated that some of these transforming genes detected by transfection represent activated cellular homologues of retroviral oncogenes<sup>7-10</sup>, although others have no known retroviral cognate<sup>7,11</sup>. To date, most of the identified transforming genes in human carcinomas have been found to be related to a member of the *ras* gene family, which encode immunologically related proteins of molecular weight (MW) ~21,000 (p21)<sup>12,13</sup>. This family is comprised of at least three members, one transduced as v-Ha-*ras* in the Harvey strain of murine sarcoma virus (Ha-MuSV)<sup>12</sup>, one as v-Ki-*ras* in Kirsten murine sarcoma virus (Ki-MuSV)<sup>12</sup>, and one identified by low-stringency hybridization to v-Ha-*ras*, termed N-*ras*<sup>14</sup>. Based on hybridization analysis using genomic DNA and the characterization of molecular clones, two structurally distinct human c-Ha-*ras* genes (c-Ha-*ras* 1 and 2) and c-Ki-*ras* genes (c-Ki-*ras* 1 and 2) have been identified<sup>15</sup>: a large version containing several introns (c-Ha-*ras* 1 and c-Ki-*ras* 2), and a smaller version lacking all (c-Ha-*ras* 2) or most (c-Ki-*ras* 1) introns. As detected by transfection of NIH 3T3 cells, the transforming genes in several human tumour cells represent activated homologues of the larger form of the c-Ha-*ras* and c-Ki-*ras* genes<sup>8-10,16-20</sup>. The role of the alternative locus (c-Ha-*ras* 2 and c-Ki-*ras* 1) in each case is unknown, although it has been reported that both c-Ha-*ras* genes of the rat can be activated *in vitro* by addition of a retroviral long terminal repeat (LTR)<sup>21</sup>.

The molecular alteration leading to activation of a transforming gene in human T24 bladder carcinoma cells has been shown to consist of a single nucleotide substitution within the first coding exon of c-Ha-*ras* 1 (refs 16-19). Efforts to localize the genetic lesion that leads to activation of the c-Ki-*ras* 2 locus have been hampered both by the difficulty in obtaining full-length Ki-*ras* 2 cDNA clones from normal and tumour cells, and by the inability to obtain clones representative of the entire locus due to its large size (>35 kilobases, kb). To determine the relationship of the two human Ki-*ras* loci with the corresponding viral transforming oncogene, and to provide a framework for an analysis of the mechanism(s) of their activation, we have molecularly cloned and sequenced all regions of

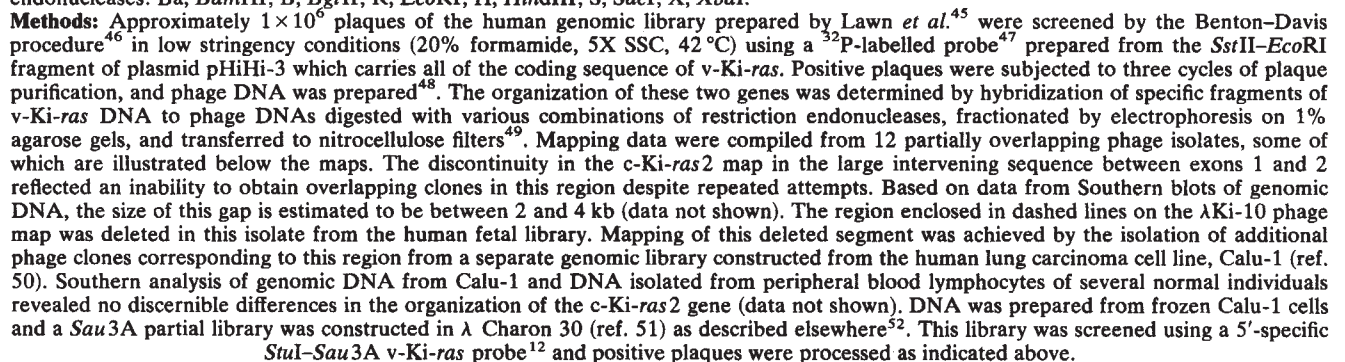
both loci sharing homology with the v-*ras* oncogene specified by Ki-MuSV.

## Molecular cloning

The two separate Ki-*ras* homologues in the human genome, termed c-Ki-*ras* 1 and c-Ki-*ras* 2, are distinguishable by Southern blot analysis, restriction endonuclease mapping and heteroduplex analysis<sup>15,22</sup>. Based on these data, Chang *et al.*<sup>15</sup> suggested that c-Ki-*ras* 1 is homologous to the corresponding v-Ki-*ras* gene, interrupted once by a small intervening sequence. The c-Ki-*ras* 2 gene appeared to be more complex, spanning a region of at least 30 kb<sup>11,20</sup>. We isolated human DNA sequences corresponding to both these loci from recombinant human DNA  $\lambda$  phage libraries using <sup>32</sup>P-labelled probes which encompassed all sequences encoding the viral p21 *ras* gene product of Ki-MuSV. Recombinant phage carrying c-Ki-*ras* 1 inserts were identified by their hybridization with probes representing both 5' and 3' portions of the v-Ki-*ras* coding sequences. The localization of these hybridizing sequences to a single 4.2-kb *Bgl*II restriction fragment confirmed this assignment<sup>20,22</sup>. Sequences of c-Ki-*ras* 2 displaying homology to v-Ki-*ras* were obtained on multiple phage clones, screened by hybridization with probes to specific regions of v-Ki-*ras*. Positive identification was provided by the presence of *Eco*RI restriction endonuclease fragments of 6.7, 3.1 and 3.0 kb after hybridization of phage DNA with the viral probe<sup>12,14</sup> and by evidence of diagnostic *Eco*RI fragments following hybridization with a probe representing the human *Alu* repeat sequences<sup>23</sup>. Orientation of the clones was determined by hybridization of phage DNA with labelled probe corresponding to the 5' or 3' region of v-Ki-*ras*, later verified by nucleotide sequence analysis. Figure 1 presents composite maps of both c-Ki-*ras* 1 and c-Ki-*ras* 2, illustrating restriction fragments hybridizing to the BLUR8 clone of human *Alu* repeat-sequence DNA and fragments that hybridize at high stringency to the v-Ki-*ras* sequences which encode p21.

## c-Ki-*ras* 1 is a pseudogene

Following identification of a phage clone containing c-Ki-*ras* 1 sequences ( $\lambda$ Ki-4), we localized the region hybridizing to the v-Ki-*ras* probe to a 1.2-kb *Bgl*II-*Hind*III restriction fragment (Fig. 1), which was then subjected to nucleotide sequence analysis. Figure 2 compares a major portion of this nucleotide sequence with the reported sequence of v-Ki-*ras*<sup>24</sup>. c-Ki-*ras* 1 clearly shares homology with sequences of Ki-MuSV that



**Fig. 2** Sequence comparison between the human c-Ki-ras1 gene and the transforming gene of Ki-MuSV. The nucleotide sequence of v-Ki-ras is from Tsuchida *et al.*<sup>24</sup>. Sequences are aligned to maximize homology. Dashes represent insertions introduced for alignment. Asterisks denote differences in single nucleotide positions. The initiator ATG for p21 ras is boxed, together with the termination codon (TAA). The first occurrence of an in-frame termination codon in the c-Ki-ras1 sequence is underlined.



encode p21. Although c-Ki-ras 1 is largely colinear with its viral homologue, numerous nucleotide substitutions, insertions and deletions are distributed throughout c-Ki-ras 1 which distort the translational reading frame. The presence of several translational termination codons in all reading frames of the gene precludes the synthesis of a functional p21 polypeptide. While c-Ki-ras 1 and its viral counterpart display overall nucleotide sequence homology of 81%, considerable divergence is evident at the 3' end of the p21 'structural' sequences. The significance of this sequence diversification became apparent only after structural analysis of the c-Ki-ras 2 locus.

### c-Ki-ras 2 encodes a 21,000-MW polypeptide

In contrast to the compact organization of the c-Ki-ras 1 locus, sequences comprising c-Ki-ras 2 span 35–40 kilobase pairs (kbp). To localize the coding regions of the gene, *EcoRI* restriction enzyme digests of the phage DNAs were hybridized to labelled sequences which encode viral Kirsten p21. Three regions were identified (Fig. 1), conforming to previous data derived from analysis of genomic DNA<sup>12,14</sup>. To verify the positions of the coding sequences, suitable restriction fragments derived from each of these regions were sequenced. Comparison of these sequences with those recently determined for the *ras* oncogene of Ki-MuSV<sup>24</sup> revealed an open reading frame of 567 base pairs (bp) spanning four exons (labelled 1, 2, 3 and 4A in Fig. 3), interrupted by intervening regions which are flanked by consensus splice junction sequences. These exons could specify a polypeptide of MW 21,660, similar to the 21,720-MW v-Ki-ras gene product. A comparison of the proteins encoded by human c-Ki-ras 2 and v-Ki-ras reveals that only six widely dispersed amino acid differences distinguish the two polypeptides, indicating that c-Ki-ras 2 represents a functional gene and suggesting that Ki-MuSV was engendered by transduction of the homologous gene in the rat.

### c-Ki-ras 1 is a processed version of c-Ki-ras 2

As is evident from a comparison of the two human Ki-ras genes, c-Ki-ras 1 resembles a processed mRNA rather than an intact gene. The large intervening sequences which disperse the p21 coding elements of c-Ki-ras 2 are absent, deleted precisely in accordance with the GT-AG splicing rules<sup>25</sup>. The c-Ki-ras 1 gene thus bears the hallmarks of a processed pseudogene, evidently generated via a mature c-Ki-ras 2 mRNA intermediate. In view of the processed nature of c-Ki-ras 1, the structural organization of this gene should reflect the splicing events which occurred in the mRNA species which served as its template. We therefore screened phage clones carrying portions of c-Ki-ras 2 for DNA homologous to sequences lying outside the 'structural' confines of c-Ki-ras 1, as well as to sequences comprising the 5'-untranslated region of human Ki-ras 2 cDNA clones (unpublished data). In this manner we determined that the region immediately 5' of the putative p21 *ras* 'coding' segment of c-Ki-ras 1 was also present ~5.7 kb upstream of the first coding exon of c-Ki-ras 2 (exon 0 in Fig. 1). Analysis of these 5'-noncoding sequences reveals two potential splice donor sites in c-Ki-ras 2 separated by 13 nucleotides, one of which was used in the mRNA precursor of the pseudogene (Fig. 4), the other of which was used in the generation of the Ki-MuSV genome (ref. 24 and our unpublished data)—this supports the interpretation that these sequences present in the pseudogene may define a 5'-noncoding exon of c-Ki-ras 2. The homology between c-Ki-ras 1 and c-Ki-ras 2 in this region continues for 99 nucleotides upstream of this presumptive splice site, at which point it terminates abruptly, possibly reflecting the 5' end of the mRNA transcript which served as the template for the pseudogene.

By using probes generated from the 3' end of the pseudogene (Fig. 1, fragments a–d), we identified a region of homology ~5.5 kb downstream of exon 4A of c-Ki-ras 2. Initially, we surmised that these 3' sequences, remote to the p21 *ras* coding segments of c-Ki-ras 2, represented a previously unidentified downstream noncoding exon. In the mRNA transcript which

served as template for the c-Ki-ras 1 pseudogene, these distal sequences had presumably undergone a splicing event which juxtaposed them to the p21 *ras* coding elements. However, examination of the nucleotide sequence (Fig. 3) revealed that homology between these sequences was not confined to the 3'-noncoding region of c-Ki-ras 1, but rather extended into the 'structural' sequences of the pseudogene (see Fig. 4). Hybridization studies and additional nucleotide sequence analysis demonstrated that homology between c-Ki-ras 1 and c-Ki-ras 2 in this region continued for >3 kb (data not shown), at which point the human DNA insert in the c-Ki-ras 1  $\lambda$  phage clone ended (Fig. 1).

### Alternative RNA splicing pathway

It was evident from an analysis of v-Ki-ras and c-Ki-ras 1 that the two genes may have evolved from distinct fourth coding exons. Comparison of the sequence of exon 4A of c-Ki-ras 2 with the sequence identified 5.5 kb downstream of this by virtue of its homology with c-Ki-ras 1 revealed a striking resemblance, implying that these two exons arose through an intragenic duplication event; we have therefore designated them exons 4A and 4B. Their relationship to each other, as well as to the corresponding exon of c-Ha-ras 1, is presented in Fig. 5. Exon 4A is highly homologous to sequences comprising part of viral Kirsten p21, while exon 4B is related to sequences within the processed pseudogene. The sequence of events envisaged for the creation of the c-Ki-ras 1 processed gene would involve a primary RNA transcript incorporating sequences spanning the entire length of the c-Ki-ras 2 locus. This RNA molecule would then have undergone the processing reactions required to excise intervening sequences. During its maturation, however, the splicing event which should join coding exon 3 and exon 4A did not occur. Instead, the 16 kb of RNA sequence corresponding to exon 4A and its flanking introns were eliminated as the 3' end of exon 3 was fused to the downstream exon 4B at its splice acceptor site. This abridged mRNA molecule apparently served as a template for reverse transcription and its DNA copy was reintroduced into the genome on a different chromosome<sup>22</sup> as c-Ki-ras 1.

This 'splicing out' of exon 4A during the generation of c-Ki-ras 1 could represent an abnormal RNA processing event which occurred in a germ cell, analogous to the aberrant transcripts of the human  $\lambda$  immunoglobulin<sup>26</sup> and mouse  $\alpha$ -globin<sup>27</sup> genes which are postulated to be precursors for their respective processed pseudogenes. Yet the conservation of coding potential exhibited by the two versions of exon 4 argues for a continued functional role, particularly in view of the divergence evident in this region of the pseudogene. As both of these exons are flanked on their 5' boundaries by a canonical splice acceptor sequence, each could conceivably contribute to the carboxy-terminal domain of the p21 *ras* protein specified by this locus. While exon 4A is capable of encoding 39 amino acids, exon 4B could encode 38 amino acids before a translational termination codon is encountered. Of these amino acids, 19 are identical, while four conservative substitutions occur. Figure 5 illustrates that the conserved regions are not distributed randomly, but rather localize to the two ends of the coding region. It is provocative that the other well characterized member of the *ras* gene family, c-Ha-ras 1, manifests a similar distribution of conserved residues.

### Discussion

We have isolated and characterized the two Kirsten-specific members of the *ras* gene family present in the human genome. Our analysis establishes c-Ki-ras 2 as a functional gene which spans ~38 kb, verifying previous size estimates based on the association of *Alu* segments with transfection of the activated version of this gene<sup>3,4,7,14</sup>. Hybridization studies of the c-Ki-ras 2 locus using probes generated from the 3' region of c-Ki-ras 1 led to the discovery of a diverged second copy of the fourth coding exon residing 5.5 kb downstream from its viral-like counterpart. Two lines of evidence support the prospect of

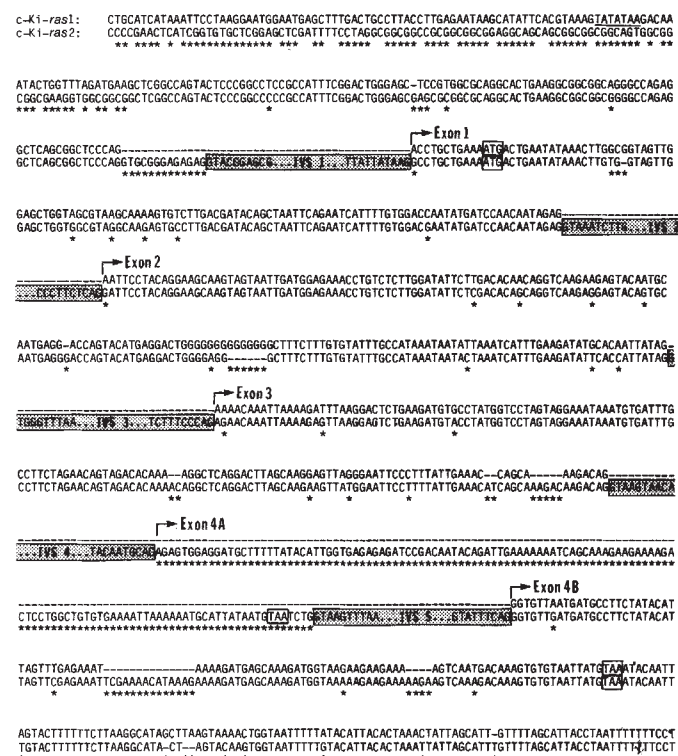
(only a single conservative amino acid difference distinguishes the two within sequences derived from exon 4A). Second, exon 4B was the contributing exon in the mRNA which ultimately served to generate the c-*Ki-ras*1 locus (Fig. 6b). Note that the presence of exon 4A in a processed message does not imply the absence of exon 4B. Indeed, it would seem that both exons

### Exon 4B

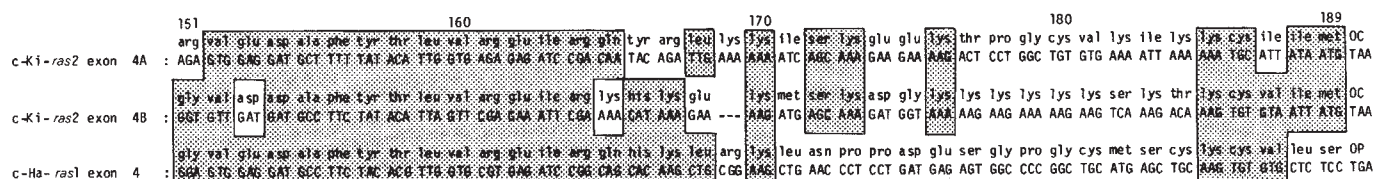
**Fig. 3** Nucleotide sequence of the human c-Ki-ras2 gene. The strategy used to determine the nucleotide sequence of the c-Ki-ras2 gene was based on the restriction mapping data presented in Fig. 1. Restriction endonuclease fragments carrying sequences which hybridized to the various probes described in Fig. 1 legend were initially subcloned into derivatives of pBR322. Detailed mapping studies allowed the isolation of overlapping restriction fragments having a maximum size of ~800 bp which were subjected to sequence analysis using the dideoxy chain termination method<sup>43</sup> following their insertion into bacteriophage M13 vectors<sup>53,54</sup>. Some fragments were additionally sequenced by the chemical method of Maxam and Gilbert<sup>44</sup>. The nucleotide sequence is numbered beginning arbitrarily at a *Sac*II site upstream of the gene. The estimated sizes of the discontinuities in the sequence within the introns are indicated. Amino acid translations of each of the four coding domains homologous to v-Ki-ras are indicated above the nucleotide sequence and are interrupted at the exon/intron junctions. The predicted amino acid sequence for exon 4B (see text) is also shown. The known RNA splice sites are marked by arrows (see Fig. 4 legend).



survived the splicing pathway in the generation of *v-Ki-ras*. This is suggested by the limited nucleotide sequence information available for this region of *v-Ki-ras*<sup>24</sup>, where 8 of 9 nucleotides following the viral sequences apparently derived from exon 4A are identical to the sequences present at the beginning of exon 4B. In addition, recently published hybridization data<sup>14</sup> indicate that a probe to the 3'-untranslated region of *v-Ki-ras* recognizes sequences in molecular clones of *c-Ki-ras2* which we have identified as being exon 4B. This structure inferred for the *Ki-MuSV* genome explains the discrepancy between our data establishing *c-Ki-ras1* as a processed pseudogene and the interpretation, based on electron microscopic analysis of DNA heteroduplexes between *c-Ki-ras1* and *v-Ki-ras*, that *c-Ki-ras1* contains one small (0.2 kb) intervening sequence<sup>15</sup>. Our interpretation is that the deletion loop observed in the heteroduplexes actually represented viral exon 4A, and not cellular intronic sequences as suggested.



**Fig. 4** Comparison of the exons of the human *c-Ki-ras2* gene and the *c-Ki-ras1* pseudogene. Nucleotide sequences are aligned to maximize homology. Dashes indicate a gap introduced for alignment. The shaded boxed regions correspond to the intervening sequences in *c-Ki-ras2*. The intron/exon boundaries in *c-Ki-ras2* were inferred from the sequence structure of the viral *Ki-ras* gene (ref. 24 and our unpublished results). The initiation and termination codons for p21 *ras* are enclosed in open boxes. Homology at the 3' end of these genes extends at least 3 kb beyond the sequence shown (see text).



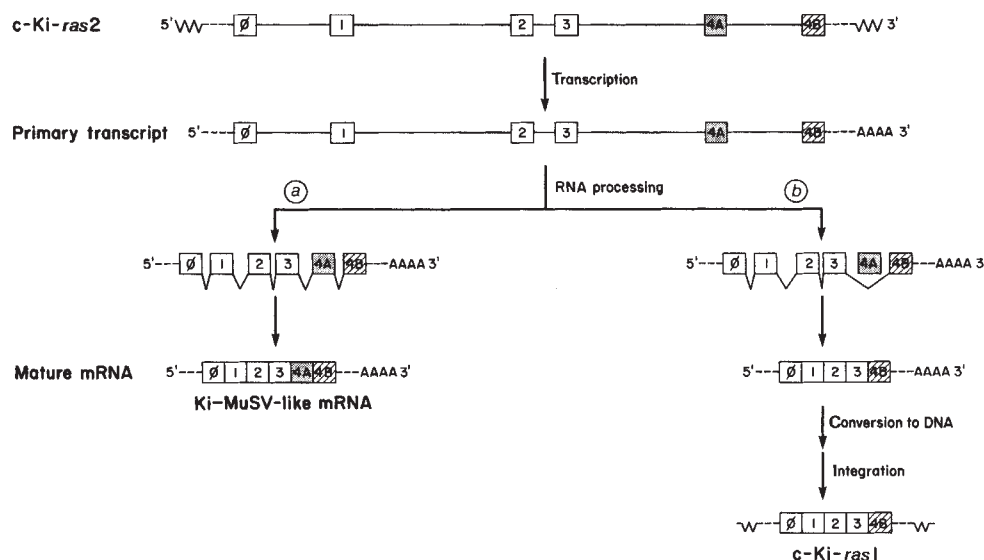
**Fig. 5** Homologies between the fourth coding exon domains of *c-Ki-ras2* (A and B), and *c-Ha-ras1*. The human *c-Ha-ras1* sequence is from Capon *et al.*<sup>19</sup>. The translated amino acid sequences are presented above the gene sequence. Shared amino acid residues are enclosed in shaded boxes. Dashes indicate insertions introduced for alignment.

The presence of two versions of the fourth coding exon has interesting implications relating to p21 expression in normal and tumour cells, in that they afford the prospect of specifying two related, but distinguishable polypeptides from a single genetic locus. Apart from a small difference in size (MW 231), a polypeptide encoded from a mRNA containing exon 4B would be distinguished by an extremely lysine-rich carboxy terminus. Direct comparison of exons 4A and 4B in Fig. 5 reveals a definitive pattern of sequence conservation and divergence which is also shared by the *c-Ha-ras1* member of the *c-ras* gene family. The region of greatest divergence among *c-Ha-ras1*, *c-Ki-ras2A* and *c-Ki-ras2B* is localized between amino acid residues 171 and 184, where all three appear to be quite unrelated. As is evidenced by the comparative protein sequence data and by homology at the nucleotide sequence level, exon 4B, although presumably representing a tandem duplicate of exon 4A, bears no greater resemblance to this exon than it does to the fourth exon of *c-Ha-ras1*. One implication of this observation is that the presence of alternative coding exons in the *c-Ki-ras2* locus is not the result of a recent event in evolutionary history, but presumably originated about the time the Harvey and Kirsten *ras* genes appeared as separate genetic loci.

Our data indicating that *c-Ki-ras2* represents a potentially functional gene capable of specifying a p21 *ras* protein sustains previous evidence that activation of the human *Ki-ras* proto-oncogene involves this locus<sup>20,34</sup>. The polypeptide predicted from this gene sequence differs from its viral homologue at only six of 189 amino acid residues, if exon 4A is utilized. Importantly, one of these differences involves the twelfth amino acid, which in the case of the viral protein is a serine<sup>24</sup> as opposed to a glycine specified by the normal human gene. Thus, the fine structure of this locus reiterates the observation that a glycine residue at position 12 of *c-Ha-ras1* specifies a normal (that is, nontransforming) gene product<sup>16-19</sup>, at least when expressed at usual physiological levels<sup>18,35</sup>. Note also that both *v-Ha-ras* and *v-Ki-ras* encode a threonine residue at position 59, which represents the site of autophosphorylation catalysed by viral p21 (ref. 13). Human *c-Ha-ras1* and *c-Ki-ras2*, on the other hand, specify an alanine residue at this position (see ref. 19 and Fig. 3). Although no information is presently available regarding the sequence of the corresponding rat genes from which the viral homologues were derived, the observation that p21 encoded by rat *c-Ha-ras1* lacks phosphothreonine<sup>13</sup> suggests that these viral phosphoacceptor sites may have arisen through a process of convergent evolution.

In striking contrast to the organization of *c-Ki-ras2*, we have found that *c-Ki-ras1* represents a functionless genetic entity, evidently the result of an ancient integration event into the germ line via a spliced RNA intermediate of a *c-Ki-ras2* transcript. Similar elements have been described in several gene systems<sup>26,27,36-39</sup>, suggesting that this reversed flow of genetic information is a relatively common occurrence in genome evolution. The homology between *c-Ki-ras1* and *c-Ki-ras2* extends for over 4 kb, most of which is downstream of the p21 structural sequences, implying that the *c-Ki-ras2* mRNA transcript which engendered *c-Ki-ras1* was at least this large and evidently possessed an extremely long 3'-untranslated region. Consistent with this interpretation is the finding that the major transcript

**Fig. 6** Alternative splicing patterns deduced for the human c-Ki-ras2 gene transcript. The extreme 5' and 3' boundaries of the primary transcript are as yet undefined and are indicated as dashed lines. Splicing pattern *a* is based on the structure of the Ki-MuSV RNA genome; the *b* pattern is inferred from the structure of the c-Ki-ras1 processed pseudogene.



of c-Ki-ras2 in both SW480 and Calu-1 human tumour cells is ~5.5 kb long (see accompanying paper<sup>40</sup>).

Although the DNA sequence demonstrates that c-Ki-ras1 is unable to express a p21 polypeptide, it is curious that sequences characteristic of mammalian promoters<sup>41</sup> can be identified just upstream of the 5' boundary of the gene (for example, the sequence TATATAA is present 29 nucleotides preceding this junction). Whether this indicates that the gene may at one time have been functional or whether it is merely coincidental is unclear. In contrast to this Kirsten *ras* pseudogene, the intronless version of the Harvey *ras* gene of the rat (c-Ha-ras2) has been reported to be active, at least when joined *in vitro* to a retroviral LTR<sup>21</sup>, although no success has been reported in activating the corresponding human gene<sup>22</sup>. Examination of the nucleotide sequence of c-Ki-ras2 reveals no clues as to where the promoter for this gene resides. This can only be indirectly inferred from size estimates of the functional gene (based on transfection analysis), analysis of the pseudogene (Fig. 4), and the localization of the 5' ends of c-Ki-ras2 cDNA clones (unpublished data), which position the promoter to a region near, or contained within, the upstream nucleotide sequence presented in Fig. 3.

Our analyses of the structure of c-Ha-ras1 (ref. 19) and c-Ki-ras2 (Fig. 3) reveal that both genes are interrupted by introns at identical positions, arguing strongly for a monophyletic origin involving the ancient duplication of a primordial *c-ras* gene. This finding suggests that the ancestor of these genes was also interrupted, consistent with the observation that the c-Ha-ras locus that contains introns is the most conserved of the *c-ras* loci: the number, size and locations of exons and introns are analogous in mice, rats and humans; the nucleotide sequences of the exons diverge very little from mouse to human (as determined by hybridization and heteroduplex analysis); and the introns are somewhat conserved, although less so than the exons<sup>15,21,42</sup>. Harvey and Kirsten *c-ras* genes display considerable organizational diversity among different species, occurring as unique loci, as duplications within the Harvey and Kirsten lineages, and as amplified gene families<sup>42</sup>. The dispersion to different chromosomes of each of the four *c-ras* genes in man<sup>22</sup> is indicative of multiple rearrangements involving these genes during evolution which served to expand and diversify the composition of this gene family.

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- Cooper, G. M. *Science* **218**, 801-806 (1982).
- Krontiris, T. G. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1181-1184 (1981).
- Murray, M. J. *et al. Cell* **25**, 355-361 (1981).
- Perucho, M. *et al. Cell* **27**, 467-476 (1981).
- Shimizu, K., Goldfarb, M., Perucho, M. & Wigler, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 383-387 (1983).
- Pulciani, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 2845-2849 (1982).
- Pulciani, S. *et al. Nature* **300**, 539-542 (1982).
- Der, C. J., Krontiris, T. G. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3637-3640 (1982).
- Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. A. *Nature* **297**, 474-478 (1982).
- Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. *Nature* **298**, 343-347 (1982).
- Lane, M.-A., Sainten, A. & Cooper, G. M. *Cell* **28**, 873-880 (1982).
- Ellis, R. W. *et al. Nature* **292**, 506-511 (1981).
- Papageorge, A., Lowy, D. & Scolnick, E. M. *J. Virol.* **44**, 509-519 (1982).
- Shimizu, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 2112-2116 (1983).
- Chang, E. H., Gonda, M. A., Ellis, R. W., Scolnick, E. M. & Lowy, D. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4848-4852 (1982).
- Tabin, C. J. *et al. Nature* **300**, 143-149 (1982).
- Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. *Nature* **300**, 149-152 (1982).
- Taparowsky, E. *et al. Nature* **300**, 762-765 (1982).
- Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. & Goeddel, D. V. *Nature* **302**, 33-37 (1983).
- McCoy, M. S. *et al. Nature* **302**, 79-81 (1983).
- DeFeo, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 3328-3332 (1981).
- O'Brien, S. J., Nash, W. G., Goodwin, J. L., Lowy, D. R. & Chang, E. H. *Nature* **302**, 839-842 (1983).
- Jelinek, W. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 1398-1402 (1980).
- Tsuchida, N., Ryder, T. & Ohtsubo, E. *Science* **217**, 937-939 (1982).
- Mount, S. M. *Nucleic Acids Res.* **10**, 459-472 (1982).
- Hollis, G. F., Hieter, P. A., McBride, O. W., Swan, D. & Leder, P. *Nature* **296**, 321-325 (1982).

- Nishioka, Y., Leder, A. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2806-2809 (1980).
- Ziff, E. B. *Nature* **287**, 491-499 (1980).
- Early, P. *et al. Cell* **20**, 313-319 (1980).
- Young, R. A., Hagenbuchle, O. & Schibler, U. *Cell* **23**, 451-458 (1981).
- Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. *Nature* **298**, 240-244 (1982).
- Crabtree, G. R. & Kant, J. A. *Cell* **31**, 159-166 (1982).
- King, C. R. & Piatigorsky, J. *Cell* **32**, 707-712 (1983).
- Sakaguchi, A. Y. *et al. Science* **219**, 1081-1083 (1983).
- Chang, E. H., Furth, M. E., Scolnick, E. M. & Lowy, D. R. *Nature* **297**, 479-483 (1982).
- Chen, M.-J., Shimada, T., Mouton, A. D., Harrison, M. & Nienhuis, A. W. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7435-7439 (1982).
- Wilde, C. D., Crowther, C. E., Cripe, T. P., Lee, M. G.-S. & Cowan, N. J. *Nature* **297**, 83-84 (1982).
- Karin, M. & Richards, R. I. *Nature* **299**, 797-802 (1982).
- Lemischka, I. & Sharp, P. A. *Nature* **300**, 330-335 (1982).
- Capon, D. J. *et al. Nature* **304**, 507-513 (1983).
- Benoist, C., O'Hare, K., Breathnach, R. & Chambon, P. *Nucleic Acids Res.* **8**, 127-142 (1980).
- Chattopadhyay, S. K. *et al. Nature* **296**, 361-363 (1982).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Maxam, A. & Gilbert, W. *Meth. Enzym.* **65**, 499-559 (1980).
- Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G. & Maniatis, T. *Cell* **15**, 1157-1174 (1978).
- Benton, W. & Davis, R. *Science* **196**, 180-182 (1977).
- Taylor, J. M., Illmensee, R. & Summers, S. *Biochim. biophys. Acta* **442**, 324-330 (1976).
- Blattner, F. *et al. Science* **196**, 161-169 (1977).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Fogh, J., Fogh, J. M. & Orfeo, T. J. *nam. Cancer Inst.* **59**, 221-226 (1977).
- Rimm, D. L., Horness, D., Kuera, J. & Blattner, F. R. *Gene* **12**, 301-309 (1980).
- Maniatis, T. *et al. Cell* **15**, 687-701 (1978).
- Messing, J., Crea, R. & Seeburg, P. H. *Nucleic Acids Res.* **9**, 301-321 (1981).
- Messing, J. & Vieira, J. *Gene* **19**, 269-276 (1982).



# Activation of *Ki-ras2* gene in human colon and lung carcinomas by two different point mutations

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*Kirsten (Ki)-ras cDNA clones were prepared from human lung and colon carcinoma cell lines expressing an activated c-Ki-ras2 gene. DNA sequence analysis and transfection studies indicate that different point mutations at the same codon can activate the gene; that most human c-Ki-ras2 mRNA uses sequences from a fourth coding exon distinct from that of its viral counterpart; and that at least one cell line is functionally homozygous for the activated gene.*

DNA-MEDIATED gene transfer techniques have provided an important approach for detecting genetic changes which comprise potentially rate-limiting steps in carcinogenesis<sup>1,2</sup>. The transfection of NIH 3T3 cells with tumour DNA has led to the identification of transforming genes in a diverse number of spontaneous carcinomas, sarcomas and haematopoietic malignancies of humans<sup>3-8</sup> as well as chemically induced tumours of other mammals<sup>3,6,9-12</sup>.

The relationship between different transforming genes detected by transfection of NIH 3T3 cells has been clarified by the recent discovery that many of these genes represent cellular homologues of the oncogene (*v-Ha-ras*) of the Harvey murine sarcoma virus or the oncogene (*v-Ki-ras*) of the Kirsten murine sarcoma virus (*Ki-MuSV*)<sup>13-16</sup>. The transforming activity associated with the T24 (EJ) human bladder carcinoma line<sup>13-15</sup> and several chemically induced mouse squamous cell carcinomas<sup>17</sup> corresponds to an activated version of the cellular homologue (*c-Ha-ras1*) of *v-Ha-ras*. Transforming genes corresponding to the cellular homologue (*c-Ki-ras2*) of *v-Ki-ras* are found in human cell lines established from bladder, lung, colon and gall bladder carcinomas, as well as biopsy tissue from lung, pancreas and colon carcinomas, and a rhabdomyosarcoma<sup>8,15,16,18</sup>.

The preceding paper shows that *c-Ki-ras2* potentially encodes two distinct proteins of molecular weight (MW) 21,000 (p21s) through differential utilization of two of its five coding exons, raising the question of whether one or both proteins have transforming activity<sup>19</sup>.

Activation of the *c-Ha-ras1* gene in T24 bladder carcinoma cells is associated with a change in one nucleotide (G→T in codon 12) giving rise to a p21 having one amino acid and a slight electrophoretic difference<sup>20-23</sup>. The presence of p21 *ras* proteins exhibiting different electrophoretic mobilities in two lung and two colon carcinoma cell lines with activated *c-Ki-ras2* genes as well as NIH 3T3 cells transformed by DNA from these tumour lines<sup>24</sup> suggests that *c-Ki-ras2* may be activated by a similar genetic lesion. To investigate these possibilities, we have cloned *c-Ki-ras2* cDNA from human lung and human colon carcinoma cell lines carrying activated *c-Ki-ras2* genes. Sequence analysis establishes that *c-Ki-ras2* mRNA transcripts are indeed derived by alternative RNA splicing and that activation has occurred via two distinct mutations of the same codon in the two cell lines. By placing the coding elements of the cloned *c-Ki-ras2* gene under the transcriptional control of a heterologous expression system, evidence is obtained to support the notion that the structural mutations observed are both necessary and in certain cases sufficient to confer transforming activity.

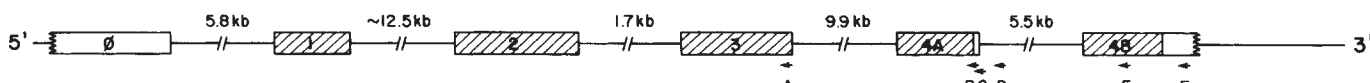
## Mutations in *c-Ki-ras2*

In contrast to the T24 bladder carcinoma oncogene (*c-Ha-ras1*), which is contained within a 4.5-kilobase pair (kbp) DNA sequence<sup>15,25</sup>, the relatively large size (>38 kbp) of the *c-Ki-ras2* locus<sup>16,18,19</sup> has hindered the molecular cloning of a contiguous segment of DNA exhibiting transforming activity from human malignancies in which this oncogene is activated. We therefore decided to clone cDNA sequences from polyadenylated RNAs of the Calu-1 lung carcinoma and SW480 colon carcinoma cell lines, which had been shown previously to contain activated *c-Ki-ras2* transforming genes<sup>16,18,24</sup>. The initial strategy of screening oligo(dT)-primed cDNA libraries prepared from total Calu-1 and SW480 poly(A) mRNA by colony hybridization using a probe corresponding to the p21 coding region of *v-Ki-ras* was unsuccessful. We therefore synthesized unique deoxyoligonucleotides complementary to *c-Ki-ras2* transcripts and used them as specific primers for cDNA synthesis.

The position and nucleotide sequence of each cDNA primer used in these experiments are shown in Fig. 1; these primers included an oligonucleotide complementary to the last five codons of the third coding exon (primer A), one overlapping the presumed p21 termination codon found in exon 4A (primer B), one corresponding to sequences immediately 3' of this termination codon (primer C), and one which hybridized 40 nucleotides further downstream (primer D). Libraries consisting of 40,000 SW480 and 10,000 Calu-1 cDNA clones prepared using primer A yielded 14 and 3 colonies, respectively, which hybridized with the *v-Ki-ras* probe. Surprisingly, libraries of 50,000 or more clones constructed in a similar manner from either SW480 or Calu-1 mRNA with primers B, C or D failed to produce any *c-Ki-ras2* cDNA recombinants.

The nucleotide sequences of the inserts of the single Calu-1 and eight SW480 cDNA clones which contained complete N-terminal coding sequences were determined by the dideoxy chain termination method<sup>26</sup> and have been compared with the coding sequences recently determined for the normal *c-Ki-ras2* gene<sup>19</sup>. Figure 2 shows that a single nucleotide in codon 12 distinguishes the three p21 coding regions. GGT, encoding glycine as amino acid 12 of the normal *c-Ki-ras2* gene product, becomes GTT, encoding valine, in SW480 cells and TGT, encoding cysteine, in Calu-1 cells.

A second nucleotide difference is found upstream of the ATG initiation codon in three of the eight SW480 cDNA clones (Fig. 2), suggesting that two distinct transforming alleles are present in the SW480 cell line. Further experimental evidence which



**Fig. 1** Strategy for cloning c-Ki-ras2 cDNAs from lung carcinoma and colon carcinoma cell lines. The map of the c-Ki-ras2 gene (from ref. 19) indicates coding (▨) and noncoding (□) regions of mature c-Ki-ras2 mRNA predicted by comparison with the sequences of the human c-Ki-ras1 pseudogene<sup>19</sup> and Ki-MuSV *ras* oncogene<sup>33</sup>.

**Methods:** The Calu-1 lung carcinoma and SW480 colon carcinoma cell lines, obtained from the American Type Culture Collection, were grown in Dulbecco's minimal essential medium containing 10% fetal bovine serum (FBS). Total RNA was extracted by the method of Berger and Birkenmeier<sup>37</sup>, polyadenylated RNA isolated on oligo(dT)-cellulose<sup>38</sup> and used to prepare cloned cDNA libraries by standard procedures<sup>39,40</sup>. The deoxyoligonucleotides used for specific priming of cDNA synthesis were prepared by the phosphotriester method<sup>41</sup> and used at a concentration of  $10 \mu\text{g ml}^{-1}$  instead of oligo(dT). The positions of the 16mers are indicated by arrows: A, dCTGTCTTGCTCTTGCT; B, dCAGATTACATTATAAT; C, dACTTAACTTACCAGA; D, dTTGCCACCTTGTTACC; E, dACCATCTTTGCTCATC; F, dCTTGTAAGTATGCC. The cDNA libraries were grown on nitrocellulose filters, the DNA from each colony fixed to the filters<sup>42</sup>, and c-Ki-ras cDNA clones identified by hybridization to a <sup>32</sup>P-labelled<sup>43</sup> probe prepared from the *Sst*II-*Eco*RI fragment of plasmid pHiHi-3 (ref. 44), which carries the v-Ki-ras coding sequence.

implicates p21 structural alterations in the activation of c-Ki-ras2 transforming ability is presented below.

Comparison of the c-Ki-ras2 gene and cDNA sequences upstream of the translation initiation codon indicates that the gene contains a 5'-untranslated exon (exon Ø of ref. 19). The splice acceptor site for the intron between exons Ø and 1 is found 11 nucleotides 5' of the translation initiation codon and the intron extends upstream for 5,800 nucleotides. The sequence of the longest cDNA clone obtained demonstrates that exon Ø spans at least 170 nucleotides. Additional experiments will be required to determine the 5' extent of this exon and whether or not additional upstream exons exist in the c-Ki-ras2 gene.

## Two p21s specified via alternative splicing

The discovery that the fourth coding exon (4A) of the c-Ki-ras2 gene is followed by a related version (4B) (ref. 19; see Fig. 1), as well as the observation that the c-Ki-ras1 processed pseudogene contains sequences homologous to exon 4B rather than exon 4A<sup>19</sup>, prompted us to design primers complementary to exon 4B sequences in order to clone any cDNAs that contained 4B sequences. Deoxyoligonucleotides were synthesized which were complementary to codons 170–174 of exon 4B (Fig. 1, primer E) and to sequences 24 nucleotides distal to the termination codon of exon 4B (Fig. 1, primer F). 40,000 clones were obtained by priming cDNA synthesis on each RNA preparation using a mixture of the two oligonucleotides; 23 and 20 independent transformants, respectively, were identified in the SW480 and Calu-1 libraries which contained all coding sequences of p21 derived from exons 1, 2 and 3. Approximately half of each set of clones contained partial exon 4B sequences beginning from primer E, while the remainder contained complete exon 4B sequences which extended to primer F. By restriction endonuclease mapping and hybridization analysis, 42 of the 43 independent recombinants were found to lack exon 4A sequences. The collection of cDNA recombinants was also screened with a probe derived from a 860-nucleotide *Rsa*I fragment containing exon 4A genomic DNA sequences as well as immediately adjacent intron sequences<sup>19</sup>, producing a single SW480 cDNA clone containing exon 4A sequences, pSW480-48, which extended from primer E (its nucleotide sequence is shown in Fig. 2).

The structure of this cDNA reflects the precise joining of exons 1–2–3–4A–4B at the consensus splice donor/acceptor sequences flanking each coding element (see ref. 19). The exon 4A–4B juncture is located four nucleotides 3' of the exon 4A termination codon, at the sequence TAATCTG/GTAAGT which closely resembles the canonical splice donor sequence, CAG/GT<sup>A</sup>AGT (refs 27, 28). Sequence analysis of several additional Calu-1 and SW480 cDNA clones initiated from the exon 4B primers (see below) confirmed that most if not all of the remaining clones reflect the splicing order of Ø–1–2–3–4B as opposed to Ø–1–2–3–4A–4B. The splicing patterns thus observed demonstrate that the c-Ki-ras2 gene directs the expression of two highly related, but distinct 21,000-

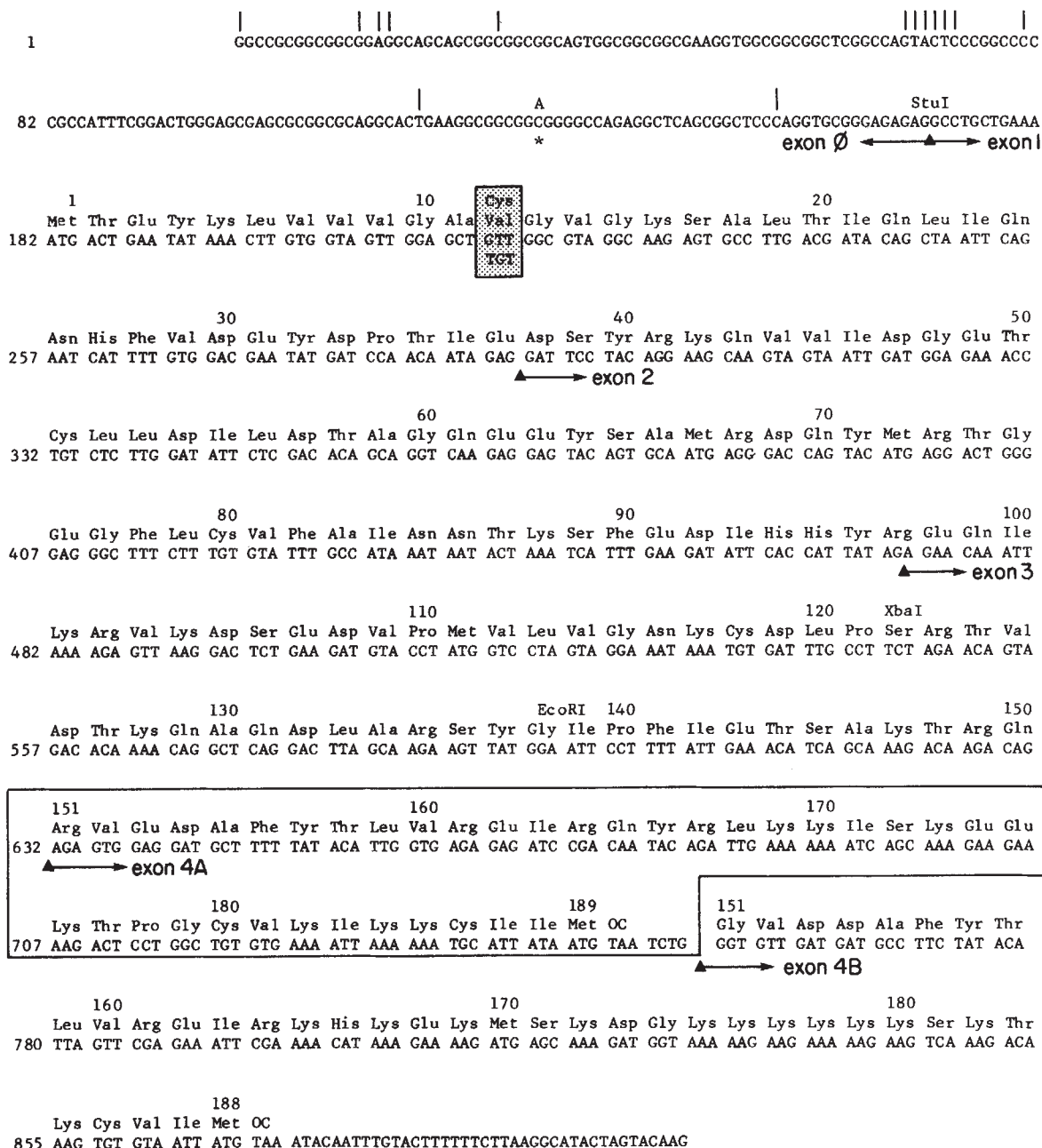
MW proteins. The proteins are identical for the 150 N-terminal residues (all but five of which are identical to those found in the rat-derived v-Ki-ras p21) which are followed either by the additional 39 residues encoded by exon 4A or the additional 38 amino acids of exon 4B (Fig. 3). While both versions of the protein exhibit highly basic carboxy-terminal regions, the localization and extreme abundance of basic amino acid residues (for example, six consecutive lysines) specified by exon 4B is especially noteworthy.

To check the possibility that priming of cDNA synthesis within exon 4B sequences had introduced a bias into the recovery of cDNA clones containing exon 4A sequences, we analysed c-Ki-ras-specific RNA sequences in the SW480 cell line with hybridization probes specific for exons 1–2–3, for exon 4A or for exon 4B. Figure 4A shows that all three probes detected a major RNA of 5.5 kilobases (kb) and one of 3.8 kb. A similar proportion of each c-Ki-ras2-specific RNA hybridized with the exon 4A (lane 2) and exon 4B (lane 3) probes, suggesting that each species contained sequences from both coding segments. Ellis *et al.*<sup>29</sup> have previously identified c-Ki-ras RNAs of 5.2 and 2.0 kb in mouse cells, each of which could be translated *in vitro* into *ras* p21. c-Ki-ras transcripts detected by the exons 1–2–3 and exon 4B probes represented ~0.05 % of polyadenylated RNA in the SW480 cell line; at least a 20-fold lower level of hybridization was observed with the exon 4A probe (compare lanes 2 and 3 of Fig. 4A). As summarized in Fig. 5, these results demonstrate that the predominant c-Ki-ras2 RNA splicing pathway in the SW480 cell line generates transcripts which encode a p21 with carboxy-terminal sequences specified by exon 4B rather than exon 4A.

To determine whether the relative abundance of exon 4A and 4B-specific transcripts varied between different cell types, a similar analysis was carried out with RNA extracted from several human tumour cell lines and normal tissues. Figure 4B shows the results obtained with the exon 4B probe. RNAs from the Calu-1 lung carcinoma (lane 3), SK-N-SH neuroblastoma, which has an activated N-ras transforming gene<sup>16</sup> (lane 4), and Detroit 562 pharynx carcinoma (lane 5) cell lines had levels of the 5.5- and 3.8-kb c-Ki-ras-specific RNA species comparable to those of SW480 (lane 2). Lower but detectable levels of each species were also seen with RNAs from the T24 bladder carcinoma cell line with activated c-Ha-ras1<sup>13–15</sup> (lane 1), as well as normal peripheral blood lymphocytes (lane 6), liver (lane 7) and secondary human embryonic kidney cells (lane 8). For each RNA, we observed a 10–20-fold lower level of hybridization with the exon 4A probe (data not shown); this suggests that the c-Ki-ras2 RNA splicing pathway is not significantly altered in cell lines having activated c-Ki-ras2 transforming genes.

Four of the five tumour cell lines examined, including two having activated c-Ki-ras2 transforming genes, had 5–10-fold higher levels of c-Ki-ras2 RNA than those observed in normal cells (Fig. 4B). These results could be significant in view of the observations that overexpression of the normal c-Ha-ras1 gene product can induce transformation of NIH 3T3 cells<sup>22,30</sup>. Further studies comparing c-Ki-ras2 RNA levels in these tumour cell lines with those found in their normal counterpart cells may shed additional light on this issue.





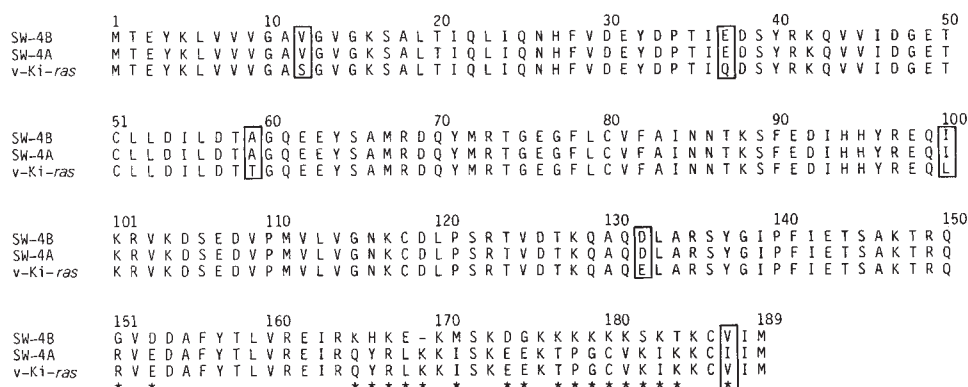
**Fig. 2** Nucleotide sequence of c-Ki-ras2 cDNAs from lung and colon carcinoma cell lines. The sequence shown is derived from the analysis of 26 independent cDNA isolates, 14 from Calu-1 and 12 from SW480-mRNAs. Sequences were determined by the chain terminator method<sup>26</sup> after subcloning suitable restriction enzyme-generated DNA fragments into phage M13 mp10 or mp11 RF DNA<sup>45,46</sup>. Internal *Xba*I and *Sma*I restriction sites are indicated and all cDNA clones were bounded by *Pst*I sites. Nucleotide numbering starts with the longest 5'-untranslated sequence obtained; nucleotide 1 corresponds to position 410 in the c-Ki-ras2 gene<sup>19</sup>. Vertical bars in the 5'-untranslated DNA sequence indicate the lengths of these sequences contained in the different cDNA clones. Multiple cDNAs began in the region between nucleotides 68 and 73 (one at 68, three at 69, six at 70, two at 71, one at 72, and three at 73). The asterisk indicates the polymorphism found in the SW480 cDNAs. The exon boundaries are derived from the alignment of c-Ki-ras2 gene and cDNA sequences. Only one cloned cDNA (pSW480-48) contained the exon 4A sequence (shown in box). All other cDNAs had exon 3 sequences spliced directly to exon 4B. The amino acid sequences predicted from the DNA sequence are shown using alternative numbering for exon 4A and exon 4B-encoded sequences. Different codons were found for amino acid 12 in cDNAs derived from the Calu-1 lung carcinoma (TGT and GGT) and SW480 colon carcinoma (GTT). The last 16 nucleotides in the figure are complementary to cDNA primer F (Fig. 1). The synthetic deoxyoligonucleotide dCTGGTGGCGTAGG was used to screen the cloned c-Ki-ras2 cDNAs to determine the number which contained GGT at codon 12 using hybridization conditions described previously<sup>47</sup>.

### Normal and transforming alleles

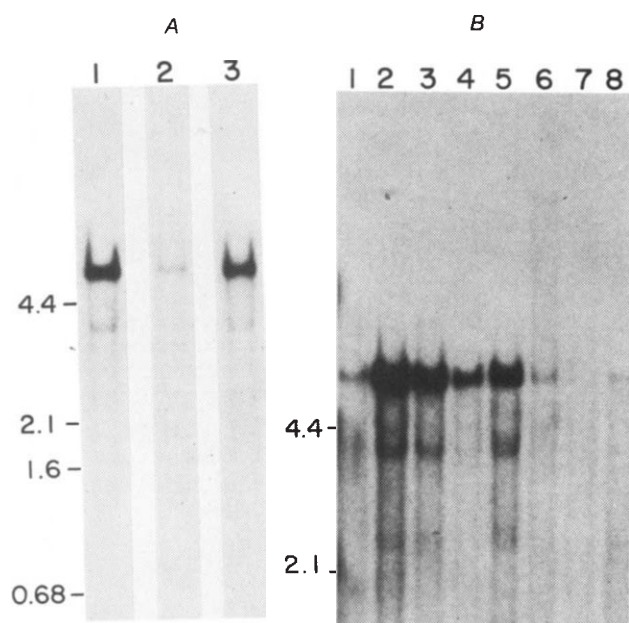
As all eight independent SW480 cDNA clones examined encoded an identically activated c-Ki-ras2 transforming gene product, and since 14 independent c-Ha-ras1 genomic DNA clones isolated from the T24 human bladder carcinoma cell line in a non-selective manner (that is, by homology to v-Ha-ras rather than by virtue of biological activity) were able efficiently to induce foci on NIH 3T3 cells<sup>22,23</sup>, it is possible that both alleles are mutated in all cases. To investigate this possibility,

the allelic composition of the c-Ki-ras2 gene in the SW480 and Calu-1 cell lines was characterized by additional sequence and hybridization analysis.

We first asked whether c-Ki-ras2 transcripts encoding a normal p21 (GGT at codon 12) could be identified in the 43 independent full-length cDNA clones by means of a synthetic deoxyoligonucleotide which overlapped amino acid codons 11–15 and corresponded to the exact sequence (dCTGGTGGCGTAGG) found in the normal c-Ki-ras2



**Fig. 3** Amino acid sequence comparison of p21s encoded by c-Ki-ras2 and v-Ki-ras. The p21 sequences labelled SW-4A and SW-4B were derived from the nucleotide sequences of cloned cDNAs obtained from SW480 colon carcinoma mRNA (See Fig. 2). 4A and 4B denote the respective exon 4A and exon 4B domains in the c-Ki-ras2 gene. Differences between the cellular (exon 4A-containing) and viral Ki-ras amino acids<sup>33</sup> are boxed. Asterisks indicate differences between exon 4A- and 4B-encoded amino acids. To allow better sequence alignment, a gap was introduced in the C-terminal part of SW-4B.



**Fig. 4** Analysis of c-Ki-ras2 transcripts in tumour cell lines and normal tissues. **A**, 5 µg of total polyadenylated RNA from the SW480 cell line were electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose paper in 20×SSC<sup>48</sup>. The resulting filter was cut into strips which were separately hybridized with radioactive probes prepared<sup>43</sup> using restriction fragments from cDNA clone SW480-48 (see Fig. 2) containing: lane 1, exons 1–3 (*StuI*–*EcoRI*, nucleotides 169–594); lane 2, exon 4A (*SfaNI*–*SfaNI*, nucleotides 632–756); lane 3, exon 4B (*SfaNI*–*RsaI*, nucleotides 756–906). Hybridizations were done in 5×SSC, 50% formamide, 10% dextran sulphate at 42 °C overnight. Filters were washed in 0.2×SSC, 0.1% SDS at 42 °C and exposed to X-ray film for 3 h for lane 1 and 42 h for lanes 2 and 3. The specific activity of the individual probes was quantitated using the dot-blot procedure<sup>31</sup> by hybridization to decreasing amounts of the plasmids containing subclones of either exon 4A or 4B from the phage DNA which carries both of these exons (λKi-59; ref. 19). **B**, 5 µg of polyadenylated RNA from various human tumour cell lines (obtained from the American Type Culture collection) and normal tissues were hybridized with the probe derived from exon 4B: lane 1, T24 bladder carcinoma line mRNA; lane 2, SW480 colon adenocarcinoma line mRNA; lane 3, Calu-1 lung carcinoma line mRNA; lane 4, SK-N-SH neuroblastoma line mRNA (3 µg); lane 5, Detroit 562 pharynx carcinoma line mRNA; lane 6, normal peripheral blood lymphocyte mRNA; lane 7, normal liver mRNA; lane 8, mRNA from a secondary culture of normal human embryonic kidney cells. The normal RNA samples were provided by William Holmes, Glenn Nedwin and Richard Lawn. The faintly hybridizing band in all lanes at 4.5 kb is probably due to the constant amounts of 28S rRNA present in all samples.

gene. This oligomer was hybridized with DNA from each recombinant by the dot-blot procedure<sup>31</sup> under conditions which favoured the annealing of precisely matched sequences (see Fig. 2). Control experiments demonstrated that the sequence of the normal gene product was readily distinguished from sequences encoding valine or cysteine residues at codon 12 which contained a single nucleotide mismatch. By this approach, we determined that three of the Calu-1 but none of the SW480 cDNAs specified a glycine 12 residue. A similar analysis of four independent clones isolated from a Calu-1 genomic DNA library<sup>19</sup>, which contained exon 1 sequences, revealed one glycine 12 sequence. Together, these results demonstrate the existence of two distinct c-Ki-ras2 alleles in each carcinoma cell line: a glycine 12 and cysteine 12 version of p21 in Calu-1, and two valine 12-encoding alleles in SW480 differentiated by a single nucleotide polymorphism in the 5'-noncoding region of the RNA (Fig. 2).

Additional nucleotide sequence analysis was performed to gain a quantitative measure of the relative abundance and number of distinct c-Ki-ras2 alleles in these carcinoma cell lines. The results are summarized as follows. All 12 SW480 cDNA clones primed from exons 3 or 4B exhibited a valine 12 residue and five of them contained the 5'-noncoding nucleotide change. Ten additional cysteine 12 and two additional glycine 12 Calu-1 cDNA clones were found to have otherwise identical nucleotide sequences.

## Transforming c-Ki-ras2 minigenes

The *in vitro* reconstitution of c-Ki-ras2 transforming gene activity from the SW480 and Calu-1 cell lines was complicated by the extended size of this locus. We therefore used an alternative approach<sup>23</sup> in which a series of plasmids was constructed so that c-Ki-ras2 coding and flanking sequences were assembled behind the simian virus 40 (SV40) early promoter and examined for their ability to induce foci on Rat-1 cells. In this way, structural features of c-Ki-ras p21 potentially critical for transforming ability could be compared at equivalent cellular expression levels.

An initial attempt to generate a c-Ki-ras2 'minigene' with transforming activity is represented as structure I in Fig. 6. However, none of the three versions (encoding valine, cysteine or glycine as amino acid 12) was able to induce morphological transformation of Rat-1 fibroblasts (<1 focus per µg of DNA). We suspected that this was due either to the constructed 'mini-intron' between exons 3 and 4A or to a lack of sequences from the coding or flanking region of exon 4B. To address the latter possibility, structure II plasmids were constructed by linearizing each structure I plasmid at a unique *Bam*HI site 2.0 kb 3' of the exon 4A translation termination codon (Fig. 6) and inserting at this site a *Bgl*II fragment from the normal c-Ki-ras2 gene encompassing the exon 4B coding region, its splice acceptor site, and 3.0 kb of additional 3' flanking sequence. The resulting set of plasmids contained two 'mini-introns', one between exons



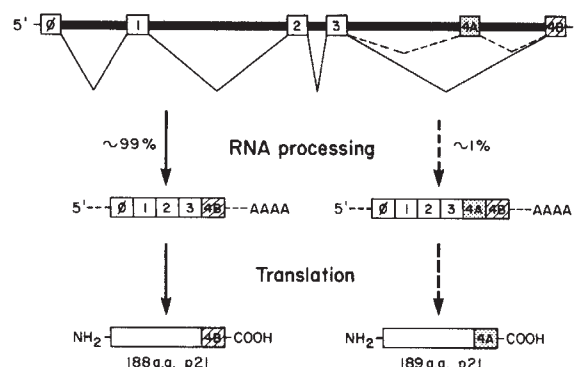
3 and 4A, and another between exons 4A and 4B. As in the previous case, sequences adjacent to the splice donors and acceptors were not altered. Plasmids having structure II which encoded either valine or cysteine, but not glycine, at codon 12 efficiently induced the transformation of Rat-1 fibroblasts on transfection (>200 foci per  $\mu\text{g}$  DNA). These results provide strong evidence that the amino acid substitutions identified in the Calu-1 lung carcinoma and SW480 colon carcinoma cell lines are required for c-Ki-ras2 gene transforming ability.

These results also suggest that the carboxy-terminal region of the transforming protein is encoded by exon 4B. Alternatively, sequences 3' to exon 4B could be necessary for efficient transcription termination and/or polyadenylation, or the presence of exon 4B in primary c-Ki-ras2 transcripts might facilitate the occasional splicing of exon 4A to exon 3 sequences, thus allowing the synthesis of an exon 4A-encoded transforming protein. To examine individually the contribution of the two alternate fourth exon coding elements to transforming activity, plasmids were constructed which placed cDNAs of the abundant splicing pathway, 1-2-3-4B (structure III), or the rare splicing pathway, 1-2-3-4A-4B (structure IV), under the transcriptional control of the SV40 early promoter. Plasmids of structure III should direct the synthesis of c-Ki-ras2 p21s having the highly basic C-terminus encoded by exon 4B, while those with structure IV should specify a version of the protein very similar to the transforming gene product of v-Ki-ras. Surprisingly, none of these plasmids exhibited transforming activity.

## Discussion

We have shown that transcripts from the activated transforming genes in human lung and colon carcinoma cell lines encode proteins that are different from one another and from the product of the c-Ki-ras2 proto-oncogene. Both differences reside at position 12 of the encoded p21s where valine or cysteine are substituted for the glycine found in the nontransforming protein, just as valine substitutes for glycine at position 12 in the T24 bladder carcinoma c-Ha-ras1-encoded p21. Single amino acid substitutions at residue 12 thus represent a common mechanism of activation of transforming activity in cellular *ras* oncogenes detected by transfection of NIH 3T3 cells. Similarly, the highly homologous transforming p21s encoded by the Harvey, BALB/c and Kirsten murine sarcoma viruses have arginine<sup>32</sup>, lysine<sup>21</sup> and serine<sup>33</sup> residues, respectively, at position 12. The mutation responsible for activation of the cellular genes has arisen from a G→T transversion whereas the three retroviral transforming genes were probably activated by G→A transitions possibly resulting from reverse transcription errors.

Which of the two polypeptides specified by the activated c-Ki-ras2 locus corresponds to the transforming polypeptide? Based on the sequence of p21 encoded by v-Ki-ras, one might expect that the protein specified by exons 1-2-3-4A represents the activated cellular counterpart of the viral transforming polypeptide, particularly as only the conservative amino acid substitution of isoleucine for valine at amino acid 187 (Fig. 3) distinguishes the cellular from the viral product of exon 4A. On the other hand, Northern analysis of c-Ki-ras2 transcripts from SW480 and other tumour cell lines demonstrates that the splice acceptor site of exon 4B is used more efficiently than that of exon 4A, indicating that most mature c-Ki-ras2 transcripts lack exon 4A sequences. The relative levels of the two processed forms of c-Ki-ras2 mRNA cannot be accurately determined due to detectable levels of cross-hybridization between these related exons, but conservative estimates indicate that the  $\emptyset$ -1-2-3-4B form is at least 20-fold more abundant than the  $\emptyset$ -1-2-3-4A-4B form in the cell types examined. This estimate is in accord with the mRNA structures inferred from isolation of cDNA clones, and consistent with the interpretation that the c-Ki-ras1 pseudogene was derived from a normally spliced c-Ki-ras2 transcript<sup>19</sup>. In this regard, it is noteworthy that sequences preceding exon 4B (TTTCAG) con-



**Fig. 5** RNA splicing pathway for c-Ki-ras2 predicted from cDNA cloning results and Northern analysis. Most of the RNA is spliced such that exon 4A is deleted from the mRNA, and a 188-amino acid p21 is encoded. A very minor amount of mRNA is produced which contains exon 4A and encodes a 189-amino acid p21. The extreme 5' and 3' ends of c-Ki-ras2 mRNA have not yet been determined.

form more closely to the canonical description of splice acceptor sites (PyPyNPYAG)<sup>27,28</sup> than sequences preceding exon 4A (ATGCAG).

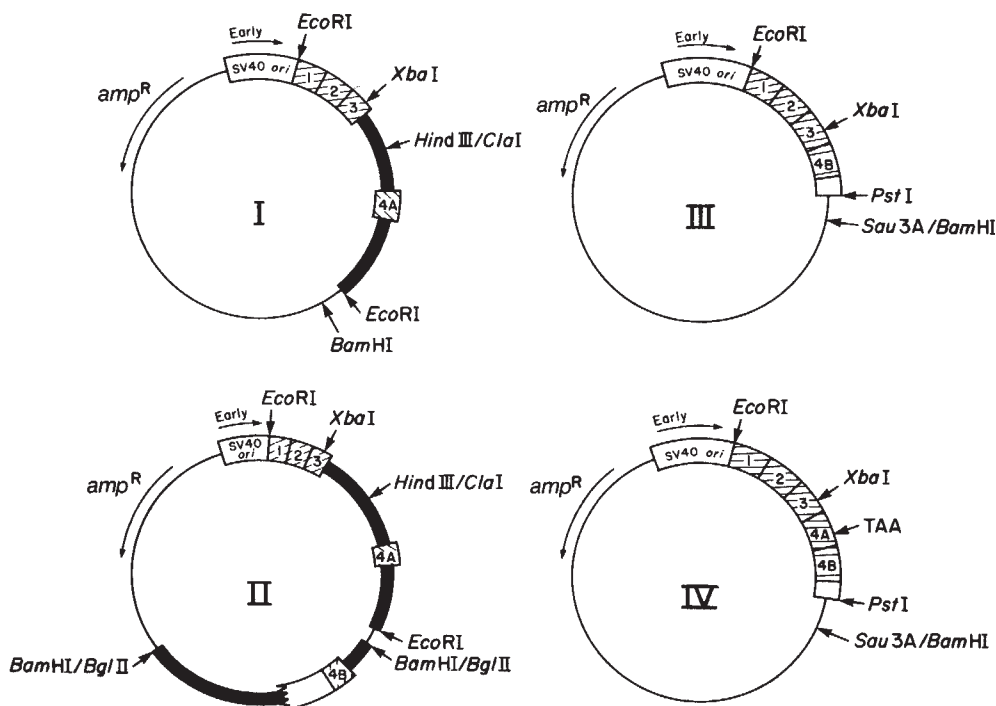
Our efforts to address which of the alternative exons specifies the transforming protein have been hampered by an inability to detect biological activity of plasmid constructions (III and IV) using either of the two versions of the c-Ki-ras2 cDNA clones, perhaps reflecting a requirement for splicing for stable mRNA formation<sup>34</sup>. Although reconstructed minigenes containing exon 4B did transform Rat-1 cells, allowing us to localize the genetic alterations associated with c-Ki-ras2 activation, it remains possible that these sequences merely provided signals necessary for the proper processing of functional RNA transcripts. Resolution of this point must await additional transfection analysis using reconstructed minigenes and/or cDNA clones.

The two examples of proto-oncogene activation studied here illustrate different ways in which an activated version of the c-Ki-ras2 locus can be preferentially expressed over its normal counterpart. In the SW480 cell line, two allelic versions of the c-Ki-ras2 locus were identified. They differ by a single nucleotide in the untranslated exon  $\emptyset$  but both bear the activating lesion and RNA transcripts of each are found in approximately equal abundance. (In view of the fivefold amplification of c-Ki-ras2 sequences reported in SW480 cells<sup>18</sup>, full interpretation of these results awaits further molecular studies.) The two alleles may have arisen by independent, yet identical mutational events; alternatively, apparent homozygosity could have resulted from gene conversion following the initial activating lesion. Given that co-conversion is frequently observed for closely linked markers in fungal systems<sup>35</sup>, it is perhaps significant that the second nucleotide difference which distinguishes the two alleles is 5,500 nucleotides from the activating lesion.

In contrast, the Calu-1 lung carcinoma cell line is heterozygous at the c-Ki-ras2 locus, harbouring both a wild type and activated allele. However, by means of an apparent bias at the transcriptional level towards expression of the activated version, it is probable that the transforming version of p21 predominates in Calu-1 cells.

While the significance of these observations remains to be determined, it is interesting to speculate that the transforming function of activated p21 proteins may be recessive to that of the normal c-ras gene product. While this notion may be called into question by the dominant behaviour exhibited by activated c-ras oncogenes in mediating the transformation of NIH 3T3 fibroblasts, we point out that transcription of endogenous c-ras genes in untransformed NIH 3T3 cells may be extremely low compared to transcription of activated human c-ras oncogenes

**Fig. 6** Plasmids constructed for DNA transfection of Rat-1 cells. Each of the four sets of plasmids (I, II, III and IV) consists of three plasmids differing only at codon 12 of *c-Ki-ras2*. SW480 cDNA clones contain a GTT (valine) and Calu-1 cDNA clones contain a TGT (cysteine) at this position. A plasmid control containing GGT (glycine) at this position was obtained by a previously described site-directed mutagenesis procedure<sup>36</sup>. Briefly, a ~500-base pair (bp) *Pst*I-*Eco*RI fragment from a SW480 cDNA clone, encoding amino acids 1-138 of *c-Ki-ras2* p21 (Fig. 2), was cloned into the M13 vector mp8 between the *Pst*I and *Eco*RI sites. A 19mer synthetic deoxyoligonucleotide, dTTGGAGCTGGTGGCGTAGG, was synthesized and used as primer for DNA repair synthesis. After transformation, resulting plaques were screened with a 13mer sequence, dCTGGTGGCGTAGG. Hybridizing plaques were sequenced to verify the mutagenesis. A 376-bp *Stu*I-*Xba*I fragment was excised from the mutagenized M13 recombinant clone for use in subsequent constructions. Plasmid set I: these three plasmids were constructed in three part ligations consisting of: (1) SV40 origin vector fragment prepared by successively digesting the plasmid p342EΔRI with *Eco*RI, filling-in to flush ends with *Escherichia coli* DNA polymerase I Klenow fragment, and digesting with *Bam*HI as described in ref. 23; (2) 376-bp *Stu*I-*Xba*I fragment from *c-Ki-ras2* cDNA clones encompassing exons 1, 2 and part of exon 3 and containing the three different codons at amino acid 12; (3) an exon 4A-containing minigene fragment. This fragment was constructed by cleaving the recombinant λ clone λ Ki-1 (ref. 19) with *Hind*III, filling-in with Klenow fragment of DNA polymerase I, and digesting with *Xba*I. A 1,400-bp fragment was isolated which extends from the *Xba*I site in exon 3 into the downstream intron. A 2,200-bp *Clal*-*Eco*RI fragment containing the entire exon 4A was purified from phage λ Ki-59 (ref. 19) following DNA polymerase I Klenow fragment repair of the *Clal* terminus. The fusion of these two fragments at their respective repaired ends generated a contiguous DNA segment which linked exon 3 with exon 4A interrupted by 1,700 bp of intervening sequence. This fragment was propagated by subcloning into an *Xba*I-*Eco*RI-ended pBR322 derivative for insertion into set I of constructions. This human DNA insert was cleaved out of the plasmid vector using *Xba*I and *Bam*HI, and therefore contains 375 bp of pBR322 sequence from *Eco*RI to *Bam*HI. Plasmid set II was constructed from plasmid set I by cleaving each member of set I with *Bam*HI and inserting a 4,900-bp *Bgl*II fragment from λ Ki-59 containing exon 4B. Plasmids having the insert in the desired orientation were determined by restriction mapping. Constructions III were obtained as follows. The three plasmids of set I were digested with *Xba*I and *Bam*HI and the large vector fragments isolated. Each was ligated to a 300-bp *Xba*I-*Sau*3A fragment from an SW480-derived *c-Ki-ras2* cDNA clone. This fragment extended from the *Xba*I site of exon 3, contained all of exon 4B and 40 bp of 3'-untranslated sequence (Fig. 2), followed by the *Pst*I site used for cDNA cloning and 63 bp of pBR322 until reaching a *Sau*3A site. Set IV of constructions was prepared similarly, except that the ~400-bp *Xba*I-*Sau*3A fragment was isolated from the plasmid pSW480-48, which also contains exon 4A. The position of the termination codon in exon 4A is indicated by TAA. All four sets of plasmids have simian virus early promoter sequences fused to the *Stu*I site of *c-Ki-ras2* cDNA (Fig. 2) 10 bp upstream of the p21 start codon. This fusion destroys the *Stu*I site, but creates an *Eco*RI site. The 380-bp *Eco*RI-*Xba*I fragment coding for amino acids 1-122 of the Ki-ras p21 was sequenced for all 12 plasmids to verify the constructions. The four sets of plasmids also contain the hepatitis B virus surface antigen polyadenylation signals and terminator sequences immediately downstream of the *Bam*HI sites<sup>49</sup>. Transformation assays were performed using Rat-1 cells as described in ref. 23.



in such cells transformed by this gene<sup>14,25</sup>. If these descriptions of loci which are potentially functionally homozygous are extended to unmanipulated human tumours, one may predict that mutations which delete or inactivate the function of *c-ras* genes may predispose an individual to the effects of activating *c-ras* lesions.

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- Weinberg, R. A. *Adv. Cancer Res.* **36**, 149-163 (1982).
- Cooper, G. M. *Science* **217**, 801-806 (1982).
- Shih, C., Padhy, L. C., Murray, M. & Weinberg, R. A. *Nature* **290**, 261-264 (1981).
- Krontiris, T. G. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1181-1184 (1981).
- Murray, M. J. *et al. Cell* **25**, 355-361 (1981).
- Lane, M.-A., Saiten, A. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5185-5189 (1981).
- Perucho, M. *et al. Cell* **27**, 467-476 (1981).
- Pulciani, S. *et al. Nature* **300**, 539-542 (1982).
- Shih, C., Shilo, B.-Z., Goldfarb, M., Dannenberg, A. & Weinberg, R. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5714-5718 (1979).
- Cooper, G. M., Okenquist, S. & Silverman, L. *Nature* **284**, 418-421 (1980).
- Shilo, B.-Z. & Weinberg, R. A. *Nature* **289**, 607-609 (1981).
- Hopkins, N., Besmer, P., DeLeo, A. B. & Law, L. W. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7555-7559 (1981).

- Der, C. J., Krontiris, T. G. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3637-3640 (1982).
- Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. A. *Nature* **297**, 474-478 (1982).
- Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. *Nature* **298**, 343-347 (1982).
- Shimizu, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 2112-2116 (1983).
- Balmain, A. & Pragnell, I. B. *Nature* **303**, 72-74 (1983).
- McCoy, M. S. *et al. Nature* **302**, 79-81 (1983).
- McGrath, J. P. *et al. Nature* **304**, 501-506 (1983).
- Tabin, C. J. *et al. Nature* **300**, 143-149 (1982).
- Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. *Nature* **300**, 149-152 (1982).
- Taparowsky, E. *et al. Nature* **300**, 762-765 (1982).
- Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. & Goeddel, D. V. *Nature* **302**, 33-37 (1983).
- Der, C. J. & Cooper, G. M. *Cell* **32**, 201-208 (1983).
- Goldfarb, M., Shimizu, K., Perucho, M. & Wigler, M. *Nature* **296**, 404-409 (1982).



26. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
27. Mount, S. M. *Nucleic Acids Res.* **10**, 459–472 (1982).
28. Perry, R. P. *J. Cell Biol.* **91**, 28s–38s (1981).
29. Ellis, R. W., DeFeo, D., Furth, M. E. & Scolnick, E. M. *Molec. cell. Biol.* **2**, 1339–1345 (1982).
30. Chang, E. H., Furth, M. E., Scolnick, E. M. & Lowy, D. R. *Nature* **297**, 479–483 (1982).
31. Kafatos, F. C., Jones, C. W. & Efstratiadis, A. *Nucleic Acids Res.* **7**, 1541–1552 (1979).
32. Dhar, R. *et al. Science* **217**, 934–936 (1982).
33. Tsuchida, N., Ryder, T. & Ohtsubo, E. *Science* **217**, 937–939 (1982).
34. Mulligan, R. C. & Berg, P. *Science* **209**, 1422–1427 (1980).
35. Holliday, R. *Genetics* **78**, 273–287 (1974).
36. Snyder, M. A., Bishop, J. M., Colby, W. W. & Levinson, A. D. *Cell* **32**, 891–901 (1983).
37. Berger, S. B. & Birkenmeier, C. S. *Biochemistry* **18**, 5143–5149 (1979).
38. Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408–1412 (1972).
39. Wickens, M. P., Buell, G. N. & Schimke, R. T. *J. biol. Chem.* **253**, 2483–2495 (1978).
40. Goeddel, D. V. *et al. Nature* **281**, 544–548 (1979).
41. Crea, R. & Horn, T. *Nucleic Acids Res.* **8**, 2331–2348 (1980).
42. Grunstein, M. & Hogness, D. S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3961–3965 (1975).
43. Taylor, J. M., Illmensee, R. & Summers, S. *Biochim. biophys. Acta* **442**, 324–330 (1976).
44. Ellis, R. W. *et al. Nature* **292**, 506–511 (1981).
45. Messing, J., Crea, R. & Seeburg, P. H. *Nucleic Acids Res.* **9**, 301–321 (1981).
46. Messing, J. & Vieira, J. *Gene* **19**, 269–276 (1982).
47. Pennica, D. *et al. Nature* **301**, 214–217 (1983).
48. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201–5205 (1980).
49. Crowley, C. W., Liu, C. C. & Levinson, A. D. *Molec. cell. Biol.* **3**, 44–55 (1983).

## LETTERS TO NATURE

### Cosmological estimate of the age of stars exploding as Type I supernovae

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There is evidence to suggest that quasars are active nuclei of galaxies most of which formed at  $z \sim 2.5$  when the age of the Universe was 1,000–2,000 Myr. During the same period of time the intergalactic gas should have formed in clusters, with a Fe abundance lower by a factor of 3 than that of oxygen. I argue here that the evolution time of the stars responsible for the synthesis of Fe nuclei (apparently supernovae Type 1, SN1) should be  $\sim 1,500$  Myr and their masses small,  $\sim 1.7\text{--}1.5M_{\odot}$ .

The nature of stars exploding as Type 1 supernovae (preSN1) is not well understood (see review by Trimble<sup>1</sup>). First there is the problem of masses of preSN1; the argument that SN1 explosions are not associated with the spiral structure of galaxies<sup>2</sup> means only that preSN1 masses should be  $< 7\text{--}8M_{\odot}$ . The conclusions that supernovae exploding in E galaxies are always SN1 seems to imply that preSN1 masses should be small because in these galaxies the star formation process, even if it had not ceased long ago, is proceeding at a very low rate, however, the latter argument is being revised (mainly for the central regions of the E-galaxies). Second, it is not clear whether SN1 outbursting in E and S galaxies are identical events.

We now try to estimate preSN1 masses using the cosmological method with the following assumptions:

(1) SN1 outbursts are the main source of Fe synthesis in the Universe. The assumption, however, is not faultless as excess Fe has never been reported in SN1 remnants<sup>3</sup>.

(2) Most quasars are active nuclei of galaxies, formed at the epoch  $z_k = 2.5\text{--}3$  when the age of the Universe was  $T_k = 1,000\text{--}2,000$  Myr (ref. 3).

(3) During the epoch of quasar formation the supernovae occurrence rate was tens of times higher than it is now. The most frequent events were the outbursts of comparatively fast-evolving stars of large mass. The high occurrence of supernovae outbursts should have drastically heated the interstellar gas which was why the gas had been 'pushed away', beyond the main bodies of galaxies and produced the hot intergalactic gas in clusters of galaxies.

Recent observations have revealed an interesting anomaly in the chemical composition of that gas. It seems that the relative abundance of Fe/O in the M87 halo is lower by a factor of 3 than in the Sun<sup>4</sup>. We explain this by assuming that Fe nuclei were being synthesized during the evolution of stars with comparatively small masses—a process which takes much longer than that of massive stars. It is also natural to relate these stars to preSN1, although, in general, our analysis does not depend on the assumption that Fe may be synthesized only in SN1 outbursts.

The anomaly in the chemical composition of the hot intergalactic gas in clusters could not be attributed to the relative excess of oxygen. It would be natural to relate this anomaly to the Fe deficiency that is observed in stars having low metallicity<sup>5</sup>. Relative to the Sun the deficiency of Fe in such stars (which undoubtedly belong to the most ancient halo population) is 3 times that of oxygen.

Note here that, according to Peimbert<sup>6</sup>, the Fe/O abundance in the planetary nebula K648, which belongs to the globular cluster M15, is about 10 times less than in the Sun.

All these anomalies in chemical composition can be explained by the following arguments. The duration of the epoch when most quasars formed is

$$\Delta T = 3/2 T_0 (1 + z_k)^{-5/2} \Delta z$$

Assuming  $z_k \sim 3$ ,  $\Delta z \sim 1$ ,  $T_0 = 16,000$  Myr gives  $\Delta T \sim 700$  Myr. For a Fe deficit to be observed in the intergalactic gas the preSN1 evolution time ( $t_1$ ) should be about  $2\text{--}3 \Delta T$ . Hence we estimate  $t_1$  as 1,500 Myr. For main-sequence stars with masses near to the solar mass  $t_{ms}$  is  $\propto M^{-4}$ . The length of time during which stars are on the main sequence,  $t_{ms}$ , is known to depend on their original chemical composition. If the latter is similar to that of the Sun ( $Y = 0.3$ ;  $z_1 = 0.04$ ), then  $t_{ms} = 7,200$  Myr (ref. 7). PreSN1 masses, with such a chemical composition, should be  $\sim 1.7M_{\odot}$ . For stars of low metallicity ( $Y = 0.3$ ;  $z_1 = 0.001$ ), then  $t_{ms} = 4,500$  Myr the preSN1 mass should be  $\sim 1.45M_{\odot}$ .

Clearly, the estimate of the mass of the stars responsible for the synthesis of Fe nuclei (most probably preSN1) depends only weakly on the model of stars adopted here, and the  $z_k$  and  $\Delta z$  values. In no case could they be stars with masses  $> 2M_{\odot}$ . Note that this conclusion agrees well with the interpretation that SN1 outbursts in E galaxies are a consequence of some kind of close binary evolution.

One may expect that for a QSO at  $z \sim 2.5\text{--}3$  the relative abundance of Fe should be low. It would be important to check this theoretical prediction by observations.

It is possible to deal with the problem of the absence of Fe overabundance in SN1 remnants by assuming a non-uniform SN1 population. One may assume, for example, that a core of radioactive  $^{56}\text{Ni}$  may form only in comparatively small-mass preSN1.

Neither should other possibilities be discarded, for example, that Fe atoms condense into liquid and solid particles in the adiabatic expansion of a supernova envelope.

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1. Trimble, V. *Rev. Mod. Phys.* **54**, 632 (1982).
2. Maza, J. & Van den Bergh, S. *Astrophys. J.* **204**, 519 (1976).
3. Shklovskii, I. *IAU Symp.* No. 104 (1982).
4. Canizares, C., Clark, G., Jernigan, I. & Markert, T. Preprint (MIT, 1982).
5. Snelten, C., Lambert, D. & Whitaker, R. *Astrophys. J.* **234**, 964 (1979).
6. Peimbert, M. *Mém. Soc. R. Sci. Liège 6th Ser.* **6**, 5, 227 (1973).
7. Mengel, L., Sweigard, A., Demarque, P. & Gross, P. *Astrophys. J. Suppl.* **4**, 733 (1979).

## Can Population III stars generate primordial helium?

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Only stars with masses in excess of  $M_c \sim 200 M_\odot$  can generate a cosmologically significant amount of helium<sup>1-3</sup> without over-producing either metals<sup>2,3</sup> or radiation<sup>2</sup>. Such very massive objects (VMOs) would also leave a large number of black hole remnants. Recent stellar evolution calculations<sup>4,5</sup> show that the helium-rich envelope of VMOs could undergo explosive ejection. We describe here a viable model of primordial helium synthesis which can be constructed after recombination, providing heavy elements are not dredged up and ejected at the same time.

A VMO with initial mass  $M_i$ , which reduces to  $M_f$  after mass loss, ejects a mass fraction of helium  $\langle \Delta Y \rangle = (1 - Y_i/2)(1 - M_f/M_i)^2$  in excess of its original amount  $Y_i$  (ref. 4). This formula is valid provided the mass loss rate is less than the critical value for which the surface shrinks at exactly the same rate as the convective core:

$$\dot{M}_{cr} = 3 \times 10^{-5} (L/L_{ED})(1 - Y_s/2)^{-2} (M/200 M_\odot) M_\odot \text{ yr}^{-1} \quad (1)$$

Here,  $Y_s$  is the instantaneous value of the surface helium abundance,  $M$  is the instantaneous star mass, and the ratio of total luminosity  $L$  to the Eddington luminosity  $L_{ED}$  is nearly unity for VMOs. Stars with mass above  $84 M_\odot$  are pulsationally unstable<sup>6</sup> and expected to have very large mass loss rates, resulting in large helium yields<sup>1</sup>. As long as  $\dot{M} < \dot{M}_{cr}$ , a VMO arranges its helium core mass to be just that required to support the surface luminosity:  $M_\alpha = M_i/(2 - Y_i)$ . The maximum possible helium yield occurs if  $M_f = M_\alpha$ :

$$\langle \Delta Y \rangle_{\max} = 0.25(1 - Y_i)^2(1 - Y_i/2)^{-1}$$

This can be obtained in two ways: (1)  $\dot{M}$  can equal  $\dot{M}_{cr}$ , so that the star remains almost completely convective; or (2) the mass loss can be less than critical throughout core hydrogen burning, but catastrophic when hydrogen is ignited in a shell. In both cases, since a black hole of mass  $\sim M_f$  forms<sup>4,5,7</sup> if the oxygen core mass ( $\sim M_\alpha$ ) exceeds  $10^2 M_\odot$ , the actual yield per mass of ejected gas is  $Y = Y_i + (1 - Y_i/2)(1 - M_f/M_i) \approx (1 + Y_i)/2$ .

Whereas possibility (1) would seem to be rather contrived, possibility (2) may be inevitable. This is indicated by our calculations<sup>4</sup> for a  $500 M_\odot$  star with Population I abundances evolving without mass loss. The ignition of  $2.5 M_\odot$  of hydrogen in a shell generated a super-Eddington luminosity which could not be convected quickly enough to avoid envelope ejection. The nuclear energy release was  $E_N = 1.3 \times 10^{52}$  ( $\Delta M_H/M_\odot$ ) ergs,  $3.3 \times 10^{52}$  ergs in our case. This is about  $L_{ED} t_{KH}$ , where  $t_{KH} \sim 10^4$  yr is the Kelvin-Helmholtz time, which is similar to the radiative diffusion time across the shell. The binding energy of the overlying layers was only  $E_B = 0.5 \times 10^{52}$  ergs, so the explosive energy released was  $E_{exp} = E_N - E_B \approx 2.8 \times 10^{52}$  ergs, corresponding to an efficiency  $\epsilon = E_{exp}/M_i c^2 \approx 3 \times 10^{-5}$ , comparable with that of a Type II supernova. Although we have not run a Population II model, we expect a similar result.

Population III stars may behave differently in detail. Stars with initially no metallicity must produce carbon through the triple- $\alpha$  reaction to catalyse the CNO reactions. They generate a CNO abundance of only  $\sim 10^{-9}$  by mass. Whereas our Population I star ignites hydrogen in the shell before core helium

burning, Population III stars ignite helium first. A strong shell-burning source will arise only if some of the oxygen generated in the core can be dredged up to catalyse it; the oxygen would then be converted to nitrogen in the shell. The subsequent dynamical evolution would be similar to that of our Population I VMO. Woosley and Weaver<sup>5</sup> have evolved a  $500 M_\odot$  Population III star and find a large dredge-up, followed by strong shell-burning which drives their envelope to very low density. However, they report no ejection. Whereas our burning and dynamics occurred over  $\sim 1 t_{KH}$ , theirs took  $\sim 6 t_{KH}$ , long enough for complete dredge-up of the helium-carbon core to deposit  $\sim 10\%$  metallicity by mass in the envelope.

The treatment of time-dependent convection, which is so crucial for this mechanism, is a major uncertainty in these calculations. The inclusion of even a small amount of rotation may have a large effect due to its coupling with convection in such radiation-dominated environments. We must regard both results as suggestive, and stress the need for further work. We will assume that a metallicity per unit mass ejected,  $\Delta Z$ , is dredged-up during this phase and lost along with the rest of the envelope. Its value is crucial to determining the possible helium yield from an early generation of VMOs. If a bare helium core is left, it will be pulsationally unstable<sup>6</sup>. We expect winds from this phase to result in further helium loss, and perhaps in metal loss as well. We ignore this contribution in the following.

We first assume that star formation proceeds continuously, so that the fractions of the Universe in gas, black hole remnants, helium, and metals gradually change as the mass fraction  $F$  of the original gas processed through stars increases. Relative to the surviving gas density, the abundances of helium ( $Y$ ), metals ( $Z$ ) and black holes ( $\phi = \rho_{bh}/\rho_g$ ) evolve according to

$$\begin{aligned} \frac{dY}{df} &= \frac{(1-Y)^2}{2(2-Y)}, & \frac{dZ}{df} &= \Delta Z \frac{(1-Y)}{(2-Y)}, \\ \frac{d\phi}{df} &= \frac{(1+\phi)}{2-Y} + \left(\frac{\dot{M}}{M}\right)_{bh} f^{-1} \phi (1+\phi) \end{aligned} \quad (2)$$

Here,  $df = (1+\phi) dF$  is the increment in the fraction of surviving gas which goes into new stars. We assume  $M_f = M_\alpha$  and that stellar evolution and chemical mixing occur sufficiently quickly to be regarded as instantaneous. The factor  $(\dot{M}/M)_{bh} f^{-1}$  is the fractional increase in the mass of the holes due to accretion over the time that a gas fraction  $df$  is processed through stars; for black holes in the  $10^2$ – $10^6 M_\odot$  range, this fraction will be small unless we invoke super-Eddington accretion rates<sup>8</sup>. We neglect the accretion which occurs during star formation and evolution, which we assume ends at a time  $t_*$ .

If we assume  $Y = Z = \phi = 0$  before star formation, equations (2) yield

$$f = \left( \frac{2Y}{1-Y} \right) - 2 \log(1-Y) \quad (3a)$$

$$Z = -2(\Delta Z) \log(1-Y) \quad (3b)$$

$$\phi = \exp\left(\frac{2Y}{1-Y}\right) - 1 \quad (t < t_*) \quad (3c)$$

$$\phi = A\phi_*(1 + \phi_* - A\phi_*)^{-1} \quad (t > t_*) \quad (3d)$$

with

$$A = M_{bh}(t)/M_{bh}(t_*)$$

For  $Y$  to be in the range 0.20–0.25,  $f$  must lie between 0.9 and 1.2, and  $F$  between 0.8 and 0.9; here we have used the approximate relation  $F = 2[1 - \exp(-f/2)]$ , which follows if we neglect the  $Y$ -dependence of  $M_f/M_i$  in evaluating  $\phi$ . Such values for  $F$  are not unreasonable in hierarchical clustering models of galaxy formation, in which pregalactic stars are the first objects to form. The associated metal production is  $Z \approx 0.5\Delta Z$ , which exceeds Population II abundances for  $\Delta Z \geq 10^{-4}$ . If  $Y = 0.25$ , the black hole abundance given by equation (3c) is  $\phi = 1.0$ . If we include accretion after star formation has



ceased, the value of  $\phi$  is given by equation (3d). In order for  $\phi$  to be large enough to provide all of the dark matter without overproducing helium, we clearly need an accretion factor  $A \geq 1.6$ ; all of the gas would be used up for  $A = 2$ .

We note that the ratio of the radiation energy density produced to that in the microwave background is<sup>2</sup>

$$\left[ 43F(1+z_*)^{-1}(1+\phi) + 10^3 \left( \frac{\epsilon_{bh}}{0.1} \right) \left( 1 - \frac{1}{A} \right) \phi (1+z_{bh})^{-1} \right] \Omega_b h^2 \quad (4)$$

where  $H = 50h \text{ km s}^{-1} \text{ Mpc}^{-1}$ ,  $\Omega_b$  is the current baryon density parameter,  $z_*$  and  $z_{bh}$  are the epochs at which most of the light is emitted from the stars and from accretion onto the holes, and  $\epsilon_{bh}$  is the efficiency with which accreted mass is turned into radiation. Even for  $F \approx 1$ , the stellar contribution need not exceed background light constraints for the typical values  $\Omega_b(1+\phi) \sim 0.1$ ,  $z_* > 10$ . With large accretion factors, the light from black holes could pose a problem if  $\epsilon_{bh}(1+z_{bh})^{-1} > 10^{-3}$ ; indeed, this has led to the suggestion that the microwave background was actually generated by black hole accretion<sup>8</sup>.

In some situations, star formation might proceed in a series of discrete steps rather than continuously. Letting  $f_n$  denote the fraction of the surviving gas which goes into stars at the  $n$ th step, and again assuming complete mixing between generations, we obtain a recursion relation for  $Y$  if  $M_i = M_\alpha$ :

$$Y_n = Y_{n-1} + 0.5f_n(1 - Y_{n-1})^2(2 - Y_{n-1} - f_n)^{-1} \quad (5)$$

Similar relations hold for  $Z$  and  $\phi$ . This formula might apply, for example, in the 'hierarchical explosions' scheme<sup>9-12</sup>, in which the shockfronts generated by the first exploding stars sweep up shells of material which fragment into more exploding stars. This can trigger a bootstrap process, with a fraction  $f_n$  of the Universe going into the  $n$ th-generation shells. We have extended this concept to include the explosions of VMOs with  $M < M_C$  during core oxygen burning<sup>2</sup>. To avoid over-enrichment, a severe constraint on the amount of explosive energy input by VMO or Type II supernovae is imposed: in the optimal case, the energy liberated can be at most  $30(Z/10^{-4}) \text{ eV}$  per baryon. The super-Eddington ejection mechanism allows high explosion efficiency without over-enrichment because all of the metals are swallowed by the black hole remnant if  $M > M_C$ , apart from the small amount released in envelope ejection. For each baryon that goes into these VMOs, the liberated energy is  $\sim 30 \text{ keV}$ .

Suppose a shell contains a mass  $M_n$  in VMOs undergoing envelope ejection at a redshift  $z_n$  which exceeds 5 (so that the expanding shock Compton cools). After the adiabatic and radiative phases of shock evolution, a shell of mass  $\xi_n M_n$  has been swept up, where<sup>2</sup>

$$\xi_n \approx 10^5 (1+z_n)^{-1.7} (\epsilon\eta/10^{-4})^{0.6} (M_n/10^6 M_\odot)^{-0.4} \quad (6)$$

Mass amplification can clearly occur over a wide range of conditions. In the next stage, a mass  $\eta\xi_n M_n$  of VMOs will explode. The fraction of the shell which goes into these VMOs,  $\eta$ , is included to illustrate the efficiency dependence and, for simplicity, is assumed to be  $n$ -independent. So long as  $z_n$  is fixed, that is, so long as the stellar and shell evolution times are shorter than the Hubble time, equation (6) implies<sup>12</sup>  $\xi_{n+1} = \xi_1^{(0.6)^n}$  for  $n \geq 1$ , which decreases towards unity as  $n$  increases if  $\eta \sim 1$ . This in turn implies that  $f_n = f_1(\xi_1/\xi_n)^{2.5}$  converges to  $f_* = f_1\xi_1^{2.5}$ , so the shell tends to some limiting mass. The enrichment is dominated by the recycling of gas through stars at this late phase. If we assume pure hydrogen initially, the helium yield becomes

$$Y_n = 0.5 \left[ 1 - \left( \frac{2-2f_*}{2-f_*} \right)^n \right] \quad (7)$$

To get  $Y = 0.25$  for large  $n$  would require  $f_n \approx 2 \ln 2 / (n + \ln 2) \approx 1.4/n$ . For example, if  $z_1 = 100$  and  $M_1 = 10^6 M_\odot$ , the first stage gives  $\xi_1 = 20$ , and  $f_1 \approx 8 \times 10^{-4}/n$  is needed.

We have raised an issue whose cosmological significance can only be decided by detailed stellar evolution calculations. If VMOs do lose their envelopes without ejecting metals, then these stars could generate much or all of the primordial helium, contribute a large number of  $10^2$ – $10^5 M_\odot$  black holes to the dark matter, and inject explosive energy into the pregalactic medium to drive galaxy formation. If envelope ejection does not occur and no metals are lost, the remnant holes could represent a larger fraction of the dark matter, but less helium would be generated. If the metal ejection exceeds  $\sim 10^{-4}$  by mass, then VMOs cannot be the source of much of the cosmological helium or the dark matter. The generation of cosmological deuterium and helium-3 by events associated with Population III stars remains an open issue.

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1. Talbot, R. J. & Arnett, W. D. *Nature* **229**, 150–151 (1971).
2. Carr, B. J., Bond, J. R. & Arnett, W. D. *Astrophys. J.* (in the press).
3. Tarbet, P. W. & Rowan-Robinson, M. *Nature* **298**, 711–715 (1982).
4. Bond, J. R., Arnett, W. D. & Carr, B. J. *Astrophys. J.* (in the press).
5. Woosley, S. E. & Weaver, T. A. in *Supernovae: A Survey of Current Research* (eds Rees, M. J. & Stoneham, R. J.) (Reidel, Dordrecht, 1982).
6. Stothers, R. & Simon, N. R. *Astrophys. J.* **160**, 1019–1029 (1970).
7. Ober, W. W., El Eid, M. F. & Fricke, K. J. *Astr. Astrophys.* **119**, 61–68 (1983).
8. Carr, B. J. *Mon. Not. R. astr. Soc.* **194**, 639–668 (1981).
9. Ostriker, J. P. & Cowie, L. L. *Astrophys. J. Lett.* **243**, L127–L131 (1981).
10. Ikeuchi, S. *Publ. astr. Soc. Jap.* **33**, 211–222 (1981).
11. Carr, B. J. & Rees, M. J. *Mon. Not. R. astr. Soc.* (in the press).
12. Carr, B. J. & Ikeuchi, S. Preprint (Kyoto University, 1983).

## <sup>182</sup>Hf chronometer for the early Solar System

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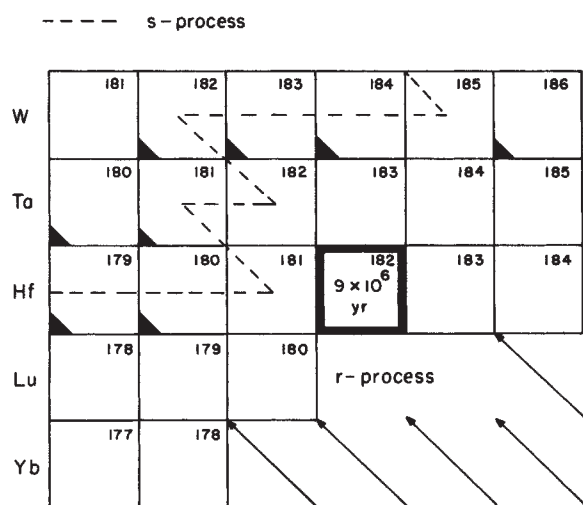
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It is now generally believed that <sup>26</sup>Al ( $t_{1/2} = 7.2 \times 10^5 \text{ yr}$ ) and <sup>107</sup>Pd ( $t_{1/2} = 6.5 \times 10^6 \text{ yr}$ ) were present in the early Solar System<sup>1</sup>. Thus, the nucleosynthetic event (supernova?) responsible for the production of these nuclei must have occurred no more than a few million years before the formation of solid bodies<sup>2,3</sup>. (However, an alternative model based on grain carriers of extinct radioactivities has also been discussed<sup>4,5</sup>.) It is possible that this event also produced the <sup>129</sup>I believed to be present in the early Solar System<sup>6-8</sup>. However, the last event to contribute <sup>244</sup>Pu to the Solar System occurred  $\sim 10^8 \text{ yr}$  before the time of solidification<sup>9,10</sup>. This latter time scale is also consistent with the lack of evidence for a <sup>247</sup>Cm chronometer<sup>11,12</sup>. We propose here that <sup>182</sup>Hf ( $t_{1/2} = 9 \times 10^6 \text{ yr}$ ) can resolve the question of whether heavy-element non-actinide nucleosynthesis occurred during the <sup>26</sup>Al-producing event. The answer to this question will help to clarify the chronology of the formation of the Solar System and will help to determine the astrophysical sites of heavy-element nucleosynthesis.

The nucleosynthesis of the nuclei from oxygen to iron (including <sup>26</sup>Al) is believed to occur in supernovae<sup>13,14</sup>. The trans-Fe nuclei are divided into two groups. Approximately half of the observed abundances of the nuclei between  $A = 80$  and  $A = 209$  can be produced through the slow (s-) neutron capture process that is thought to occur in the He-burning zones of red giant stars<sup>15</sup>. To explain both the other half of the observed abundances of the heavy nuclei and the existence of the actinides, the rapid (r-) neutron capture process is required<sup>15</sup>. Despite much research on this subject, the astrophysical site of the r-process is still not known<sup>16</sup>.



**Fig. 1** Expected paths of the slow (s) and rapid (r) neutron-capture process near  $A = 182$ . Stable nuclei are indicated by shaded triangles. All of the  $A = 182$  r-process yield passes through the  $9 \times 10^6$  yr  $^{182}\text{Hf}$  reaching the only stable  $A = 182$  isobar,  $^{182}\text{W}$ .

In the standard r-process, neutron fluxes are so high that Fe-group nuclei can be processed all the way up to the actinides. However, recently developed models suggest that there may be less dramatic events which synthesize many nuclei (but perhaps not the actinides) normally ascribed to the r-process<sup>17-19</sup>. Such alternative processes tend to occur in shock-heated zones of supernovae adding neutrons to Fe-group and s-process nuclei produced in previous stellar generations. While it is known that an actinide-producing r-process did not occur in the  $^{26}\text{Al}$  event, it is possible that one of these alternatives to the standard r-process did. To test this idea, it would thus be desirable to find a short-lived r-process only nucleus heavier than  $^{107}\text{Pd}$  and  $^{129}\text{I}$  but lighter than  $^{244}\text{Pu}$  and  $^{247}\text{Cm}$  that could have been present in the early Solar System.

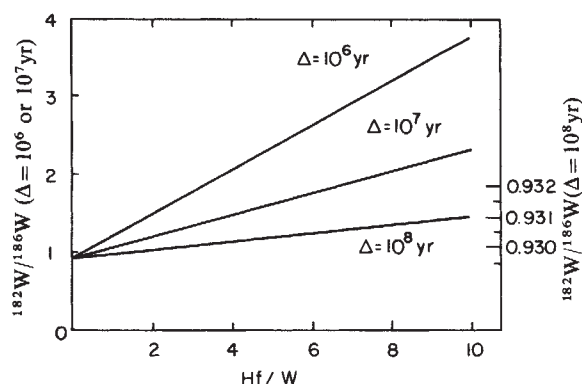
As can be seen in Fig. 1, the  $t_{1/2} = 9 \times 10^6$  yr  $^{182}\text{Hf}$  is such a nucleus. The instability of  $^{181}\text{Hf}$  prevents the s-process from reaching  $^{182}\text{Hf}$ . Furthermore, all of the  $A = 182$  yield from the r-process will pass through  $^{182}\text{Hf}$  on its way back to the only stable  $A = 182$  isobar,  $^{182}\text{W}$ . With a half life of only  $9 \times 10^6$  yr, any  $^{182}\text{Hf}$  present at the time of solidification will have long since decayed to  $^{182}\text{W}$ . Thus, the signature of live  $^{182}\text{Hf}$  in the early Solar System would be the presence today of material containing  $^{182}\text{W}$  isotopic abundance excesses which vary linearly with the  $\text{Hf}/\text{W}$  elemental abundance ratios.

The suggestion that  $^{182}\text{Hf}$  might have been present in the early Solar System was first made by Merz<sup>20</sup>, and later discussed by Schramm<sup>21</sup> and by Clayton<sup>22</sup>, but no estimates were given for the size of the  $^{182}\text{W}$  anomalies that might be observed today. Furthermore, when this suggestion was first made, there was little experimental evidence for the presence of any short-lived radioactivities in the early Solar System. Thus, to our knowledge, no studies have been made of the W isotopic abundances in meteorites.

To estimate the size of the  $^{182}\text{W}$  anomalies that might be observed today, one needs to know the amount of  $^{182}\text{Hf}$  (compared with the amounts of other stable Hf isotopes) that is expected to be produced in the r-process. Then if we compare the present  $^{182}\text{W}$  abundance to that of the r-process only  $^{186}\text{W}$ , we find

$$\frac{^{182}\text{W}}{^{186}\text{W}} = \left[ \frac{^{182}\text{W}}{^{186}\text{W}} \right]_{\text{cc}} + \left[ \frac{P(182)}{P(180)} \right] \left[ \frac{^{180}\text{Hf}_r}{\text{Hf}} \right] \left[ \frac{\text{W}}{^{186}\text{W}} \right] \left[ \frac{\text{Hf}}{\text{W}} \right] f_r(\Delta) e^{-\lambda \Delta} \quad (1)$$

where  $(^{182}\text{W}/^{186}\text{W})_{\text{cc}}$  = average Solar System abundance ratio;  $P(180)$  and  $P(182)$  are the r-process production rates for  $A =$



**Fig. 2** Expected  $^{182}\text{W}/^{186}\text{W}$  isotopic abundance ratios as a function of the  $\text{Hf}/\text{W}$  elemental abundance ratios for three different values of  $\Delta$ . All of the results are based on an assumed value of  $P(182)/P(180) = 1/2$  and  $f_r(\Delta) = 1$ . Note that the left-hand vertical scale is to be used for the  $\Delta = 10^6$  yr and  $\Delta = 10^7$  yr lines, while the right-hand scale applies to the  $\Delta = 10^8$  yr line.

180 and  $A = 182$ , respectively;  $^{180}\text{Hf}_r$  is the r-process component of the observed  $^{180}\text{Hf}$  abundance;  $\text{Hf}/\text{W}$  is the elemental abundance ratio in the sample;  $\lambda$  is the  $^{182}\text{Hf}$  decay constant;  $f_r(\Delta)$  is the fraction of Solar System r-process nuclei synthesized in the  $^{182}\text{Hf}$ -producing event; and  $\Delta$  is the time interval between this event and the formation of solid bodies. Several different studies of r-process nucleosynthesis<sup>16,17,19,23</sup> suggest that  $P(182)/P(180)$  is in the range 0.5–2. The observed Solar System values for the other quantities required in equation (1) are:  $(^{182}\text{W}/^{186}\text{W})_{\text{cc}} = 0.9296$ ,  $(^{180}\text{Hf}/\text{Hf}) = 0.3524$ , and  $(^{186}\text{W}/\text{W}) = 0.2841$  (ref. 24).  $^{180}\text{Hf}_r$  can be calculated by subtracting the s-process contribution to  $^{180}\text{Hf}$ . Using their fitted value of  $\sigma N_s$  and their measured  $^{180}\text{Hf}$  neutron capture cross-section, Beer *et al.*<sup>25</sup>, obtain  $^{180}\text{Hf}_r/^{180}\text{Hf} = 0.49$ .  $f_r(\Delta)$  is a difficult quantity to estimate. From the measured  $^{26}\text{Al}/^{27}\text{Al}$  ratio at the time of solidification, and estimates of the  $^{26}\text{Al}/^{27}\text{Al}$  production ratio, it has been inferred that the dilution factor for material contributed by the  $^{26}\text{Al}$ -producing event was small<sup>1</sup> [that is,  $f(\Delta) \geq 10^{-1}$ ]. Thus if this event also produced  $^{182}\text{Hf}$ , one might expect  $f_r(\Delta) \geq 10^{-1}$ . However, from the measured  $^{129}\text{I}/^{127}\text{I}$  ratio at the time of solidification and standard r-process estimates of the production ratio of these two isotopes, a value of  $f_r(\Delta) \approx 10^{-4}$ – $10^{-3}$  has been inferred<sup>1</sup>. Note, however, that we are discussing non-standard r-processes which may produce abundance distributions very different from the observed Solar System r-process abundances. Thus, the low  $^{129}\text{I}/^{127}\text{I}$  ratio may reflect low  $^{129}\text{I}$  production in this last event rather than a large dilution factor<sup>2</sup>. Therefore, for the sake of discussion, we have taken  $f_r(\Delta) = 1$  in our calculations. Thus one may calculate the expected present  $^{182}\text{W}/^{186}\text{W}$  isotopic abundance ratio as a function of the  $\text{Hf}/\text{W}$  elemental abundance ratio and of  $\Delta$ . The results of this procedure for assumed values of  $P(182)/P(180) = 1/2$ ,  $f_r(\Delta) = 1$ , and values of  $\Delta$  of  $10^6$ ,  $10^7$  and  $10^8$  yr are shown in Fig. 2.

As can be seen in Fig. 2, if the event which produced  $^{26}\text{Al}$  and  $^{107}\text{Pd}$  also produced  $^{182}\text{Hf}$ , and if  $f_r(\Delta) \approx 1$ , then very large  $^{182}\text{W}$  abundance excesses are expected. However, even if  $f_r(\Delta)$  is small, a value of  $\Delta \leq 10^7$  yr will still lead to observable  $^{182}\text{W}$  excesses. For large values of  $f_r(\Delta)$ , even if the last  $^{182}\text{Hf}$ -producing event occurred  $\sim 10^8$  yr before solidification,  $^{182}\text{W}$  excesses should still be detectable. The observation of such  $^{182}\text{W}$  abundance excesses will help to determine the correct value of  $f_r(\Delta)$ . The problem now is to locate meteoritic samples with high and varying  $\text{Hf}/\text{W}$  abundance ratios. The fine-grained Allende inclusions studied by Grossman and Ganapathy<sup>26</sup> show enrichments of the lithophilic refractory elements by factors of up to 10 relative to the siderophilic refractory elements. Since Hf is lithophilic while W is siderophilic<sup>27</sup>, this type of inclusion



seems to be a good place to search for this type of  $^{182}\text{W}$  isotopic anomaly. However, the absolute Hf and W abundances in such inclusions are depleted relative to the average of CI chondrites. Thus, such experiments will require sensitivities to very small W abundances.

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1. Wasserburg, G. J. & Papanastassiou, D. A. in *Essays in Nuclear Astrophysics* (eds Barnes, C. A., Clayton, D. D. & Schramm, D. N.) 77–140 (Cambridge University Press, 1982).
2. Cameron, A. G. W. & Truran, J. W. *Icarus* **30**, 447–461 (1977).
3. Schramm, D. N. in *Protostars and Planets* (ed. Gehrels, T.) 384–398 (University of Arizona Press, 1978).
4. Clayton, D. D. *Astrophys. J.* **199**, 765–769 (1975).
5. Clayton, D. D. *Space Sci. Rev.* **24**, 147–226 (1979).
6. Reynolds, J. H. *Phys. Rev. Lett.* **4**, 8–10 (1960).
7. Hohenberg, C. M., Podosek, F. A. & Reynolds, J. H. *Science* **156**, 233–236 (1967).
8. Podosek, F. A. *Geochim. cosmochim. Acta* **34**, 341–365 (1970).
9. Rowe, M. W. & Kuroda, P. K. *J. geophys. Res.* **70**, 709–714 (1965).
10. Alexander, E. C. Jr, Lewis, R. S., Reynolds, J. H. & Michel, M. L. *Science* **172**, 837–840 (1971).
11. Chen, J. H. & Wasserburg, G. J. *Geophys. Res. Lett.* **7**, 275–278 (1980).
12. Chen, J. H. & Wasserburg, G. J. *Earth planet. Sci. Lett.* **52**, 1–15 (1981).
13. Schramm, D. N. & Arnett, W. D. *Explosive Nucleosynthesis* (University of Texas Press, Austin, 1973).
14. Woosley, S. E. & Weaver, T. A. in *Essays in Nuclear Astrophysics* (eds Barnes, C. A., Clayton, D. D. & Schramm, D. N.) 377–399 (Cambridge University Press, 1982).
15. Burbidge, E. M., Burbidge, G. R., Fowler, W. A. & Hoyle, F. *Rev. mod. Phys.* **29**, 547–650 (1957).
16. Norman, E. B. & Schramm, D. N. *Astrophys. J.* **228**, 881–892 (1979).
17. Blake, J. B. & Schramm, D. N. *Astrophys. J.* **209**, 846–849 (1976).
18. Thielemann, F. K., Arnould, M. & Hillebrandt, W. *Astr. Astrophys.* **74**, 175–185 (1979).
19. Cowan, J. J., Cameron, A. G. W. & Truran, J. W. *Astrophys. J.* **265**, 429–442 (1983).
20. Merz, E. *Geochim. cosmochim. Acta* **26**, 347–349 (1962).
21. Schramm, D. N. *A. Rev. Astr. Astrophys.* **12**, 383–406 (1974).
22. Clayton, D. D. *Astrophys. J.* **224**, 1007–1012 (1978).
23. Schramm, D. N. *Astrophys. J.* **185**, 293–301 (1973).
24. Cameron, A. G. W. in *Essays in Nuclear Astrophysics* (eds Barnes, C. A., Clayton, D. D. & Schramm, D. N.) 23–43 (Cambridge University Press, 1982).
25. Beer, H., Käppeler, F. & Wisshak, K. *Astr. Astrophys.* **105**, 270–277 (1982).
26. Grossman, L. & Ganapathy, R. *Geochim. cosmochim. Acta* **40**, 967–977 (1976).
27. Amiruddin, A. & Ehmann, W. D. *Geochim. cosmochim. Acta* **26**, 1011–1022 (1962).

## Solar oscillations with 13-day period

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Claverie *et al.*<sup>1</sup> have recently discussed solar observations made over a 3-month period in the summer of 1981 which show oscillatory velocity with 13.1-day period and  $6.6 \text{ m s}^{-1}$  amplitude. Among alternative explanations they reject the possibilities that they see the Doppler shift from a radial oscillation, because the amplitude is implausibly large, and that their signal was induced by solar magnetic fields, as typical mean solar fields are too small. We have examined photoelectric drift-scan measurements of the solar diameter and full-disk magnetograms taken at Kitt Peak National Observatory for evidence of variations corresponding to the velocity oscillations of the 13.1-day period. We report here an upper limit on radius variations which is a factor of six below the amplitude needed to explain the velocity observations as a radial oscillation and we also consider the possible role of the rotation of large-scale surface magnetic features.

As part of a programme to measure the solar diameter<sup>2</sup> with the Kitt Peak Vacuum Telescope and Diode Array Magnetograph<sup>3,4</sup>, numerous measurements were obtained during the above 1981 period. We use the classic drift-scan technique

wherein the Earth's rotation sweeps the Sun's image across a fixed telescope and detector. Knowing the rotation rate of the Earth and the Earth–Sun distance, we derive the angular diameter of the Sun from the time difference between limb passages.

The observations were made with one of the 512-element diode arrays at the Kitt Peak Vacuum Telescope; this detector was inclined with respect to the drift direction of the solar image so that the limb-to-limb intensity variation was recorded along a series of chords spaced over  $\sim 0.25 R_{\odot}$  on either side of the solar equator. Several such measurements were made on each of the days we observed, and the averages of these form the basis for the work discussed here.

Observations were made on 33 days between 18 February and 10 September 1981 with an average separation between observations of 6.4 days. Unexplained (possibly non-solar) day-to-day variation at a r.m.s. level of  $3.6 \times 10^{-4} R_{\odot}$  in the radius is present in our data, and the spacing of the observations is not ideal for Fourier analysis. However, the data are frequent and precise enough that a 13.1-day oscillation of  $1.7 \times 10^{-3} R_{\odot}$  amplitude in the solar radius (which would be required to explain the velocity data<sup>1</sup>) would be readily detectable. In fact, no power above the noise level could be discerned in our data at this frequency, and we set an upper limit of  $2.6 \times 10^{-4} R_{\odot}$  for the amplitude of an harmonic component with 13.1-day period. We have also examined power spectra of the visual drift-scan measurements reported by Wittman *et al.*<sup>5</sup> for the same period and find their results consistent with this upper limit.

Claverie *et al.*<sup>1</sup> rejected the possibility that their observed signal was due to residual sensitivity to Zeeman splitting of the solar potassium line combined with changing magnetic field patterns on the Sun. However, several others<sup>6–8</sup> have suggested that the observed signal was induced by the rotation of solar features such as sunspots or active regions, since a local inhomogeneity in the slope of the potassium line wavelength profile will lead to a variable integrated-light velocity signal as the structure crosses the disk (see ref. 7 for details).

We considered the related possibility that a correlation between the strengths of the solar potassium line and magnetic field might change the average wavelength of the line as magnetic patterns rotate across the disk. An examination of daily full-disk magnetograms made at Kitt Peak shows a surprising similarity of magnetic field patterns every 13 days during the summer of 1981 which makes this hypothesis at least plausible. To test the idea quantitatively, the absolute values of the magnetic flux measurements were weighted according to their disk position by an assumed rotation law and integrated to synthesize an apparent full-disk velocity signal under the assumption that the potassium line shape varies linearly with magnetic flux. Note that the amplitude of the synthesized signal is unnormalized as no attempt was made to calibrate this relationship. The results show a variation with time that roughly matches Claverie *et al.*'s observations; however, as shown in Fig. 1, a Fourier transform of this synthesized signal shows equally strong peaks at periods of 27 and 13 days as well as weaker peaks at higher harmonics of a solar rotation period,

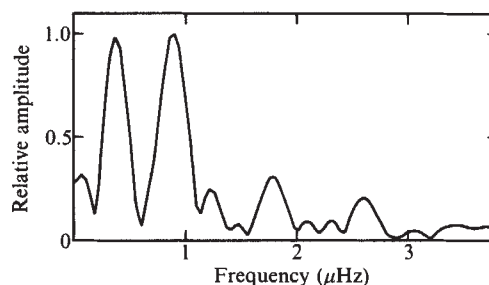


Fig. 1 The amplitude of the Fourier transform of the integrated-light velocity signal synthesized from Kitt Peak magnetograms. The time period covered is the same as in ref. 1.

whereas the velocity data show only a single peak at about a 13-day period. A possible explanation of this discrepancy is that the relation between field strength and potassium line shape may be nonlinear so that our weighting procedure induces harmonic distortion in the transformed amplitudes. We are presently obtaining and analysing new potassium line data with the Vacuum Telescope to investigate this possibility and to determine whether better calibrated predictions may be made from our magnetograms.

The distribution of amplitude in Fig. 1 agrees qualitatively with the results of Edmunds and Gough<sup>6</sup> which were derived from different data and synthetic line profiles. However, Durrant and Schröter<sup>7</sup> synthesize a signal from an assumed relation between the potassium line profiles and plage index which, like the velocity data, has a much weaker amplitude at the rotation period than at the first harmonic. Similarly, Andersen and Maltby<sup>8</sup> are able to derive from sunspot data alone a signal remarkably like the observed one.

Our results confirm that the reported oscillatory velocity of 13.1-day period is not a simple radial oscillation. The magnetogram data may be consistent with a velocity signal induced by localized inhomogeneities rotating across the disk, but we believe that better observational calibration of the potassium line response to various classes of solar features is needed to determine how much of Claverie *et al.*'s results are attributable to surface rotation.

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1. Claverie, A. *et al.* *Nature* **299**, 704–707 (1982).
2. Duvall, T. L. Jr & Jones, H. P. in *Variations of the Solar Constant* (ed. Sofia, S.) 129–130 (NASA, Washington DC, 1981).
3. Livingston, W. C. *et al.* *Appl. Opt.* **15**, 33–39 (1976).
4. Livingston, W. C., Harvey, J., Slaughter, C. & Trumbo, D. *Appl. Opt.* **15**, 40–52 (1976).
5. Wittman, A. D., Bonet Navarro, J. A. & Wohl, H. in *The Physics of Sunspots* (eds Cram, L. E. & Thomas, J. H.) 424–433 (Sacramento Peak Observatory, Sunspot, 1981).
6. Edmunds, M. G. & Gough, D. O. *Nature* **302**, 810–812 (1983).
7. Durrant, C. J. & Schroter, E. H. *Nature* **301**, 589–591 (1983).
8. Andersen, B. N. & Maltby, P. *Nature* **302**, 808–810 (1983).

## New measurements of the solar diameter

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According to standard solar models, the energy radiated from the surface of the Sun is equal to that generated by purely nuclear processes in the core. In the absence of perturbations, structural changes take place only on the nuclear evolutionary time scale; for example, during the last  $10^8$  yr, the Sun's semi-diameter would be expected to have increased by  $\sim 4$  arc s (ref. 1). Claims based on meridian transit measurements that the Sun has been shrinking on a much shorter time scale<sup>2</sup>,  $\sim 1$  arc s per century<sup>-1</sup>, and thereby changing its potential energy, have been disputed and upper limits on any secular decrease have been shown to be consistent with a constant diameter<sup>3,4</sup>. Measurements made during the 31 July 1981 total eclipse, reported here, together with a re-analysis of previous eclipse and Mercury transit measurements, confirm that there is no evidence for any secular change in the solar diameter, with a reduced upper limit. However, there is increased support for an  $\sim 80$ -yr cyclic variation.

Previously, attention was drawn to two methods of measuring the solar diameter using observations made intermittently over the past 250 yr. These methods used timings of transits of the planet Mercury across the disk of the Sun<sup>3,4</sup> and timings of the durations of total solar eclipses<sup>4</sup>. For the latter case, it was noted that there were no suitable timings in the literature after 1925. It was partly to rectify this situation, as well as to investigate the errors involved in timing measurements, that an expedition was mounted by members of University College London (UCL) to observe the 31 July 1981 total eclipse from Eastern Siberia, USSR<sup>5</sup>.

The path of totality stretched from the Black Sea to the Northern Pacific Ocean, and the eclipse was successfully observed by the UCL group from a site at  $55^\circ 57' 54'' \pm 2''$ N,  $101^\circ 24' 21'' \pm 4''$ E, 391 m above mean sea level near Bratsk, Eastern Siberia, where two independent timings were made with digital chronographs. Timing commenced at second contact, defined as the instant at which the last Bailey's bead was judged to have disappeared, and was stopped at third contact, when the first bead of light reappeared. Observations were made by eye in order to reproduce the way in which most of the previous observations were made, thus avoiding the possibility of introducing systematic errors. The durations recorded were: 107.97 s by J. H. Parkinson; 107.55 s by E. J. Breeveld giving a mean of 107.76 s.

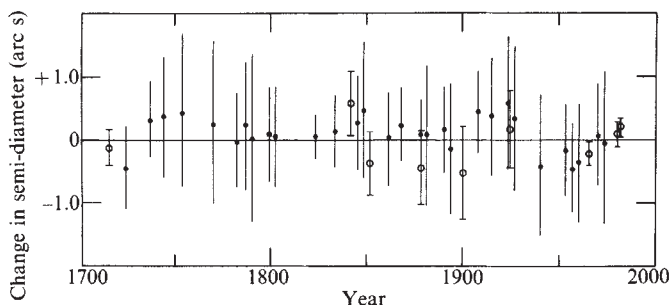
It is not possible to assign error bars to this mean value in the same way that the standard deviation was calculated for the historical measurements where several (typically 15) durations were reported for each eclipse. Instead, the range of possible values is used in the analysis that follows. From photographs taken at 1-s intervals around second and third contacts with a motorized 35-mm camera and telephoto lens, this range of values is restricted to  $\pm 0.5$  s about the mean value.

After taking into account the location of the site and the topography of the lunar limb<sup>6</sup>, the observed eclipse duration leads to a deviation,  $\delta R$ , of the Sun's semi-diameter of  $+0.20 \pm 0.15$  arc s from the standard value of 959.63 arc s. This point is plotted in Fig. 1, together with the previous results from ref. 4 and a measurement from the 1980 total eclipse which will be discussed later. The error bar on the 1981 measurement, although estimated conservatively above, appears considerably smaller than most of the previous eclipse measurements and it is unlikely that improvements in the timing equipment are solely responsible. It is probable that many previous observers have been uncertain about exactly what they should be timing, and some observers may have measured from a time just before second contact, when the remaining arc of sunlight often breaks into beads, to just after third contact, when the opposite occurs. Other observers may have been confused by the scattered light that is present just after second contact and, again, before third contact. Such problems were obviated for the 1981 eclipse by adopting the contact definitions given above and making very detailed predictions of the bead structure expected around second and third contact to avoid confusion. In this way any elements of surprise were removed, as the observers knew in advance broadly what they were likely to see and so could concentrate on making the required timings.

Two independent timings of the 16 February 1980 eclipse made in Kenya have been reported<sup>7</sup>. After analysis, a mean deviation  $\delta R = +0.09$  arc s was obtained, with a range of  $\pm 0.20$  arc s, which agrees with the 1981 measurements well within the error bars. It is therefore safe to conclude that no inconsistencies have been introduced by one observer using binoculars and the other working from a projected image.

Parkinson, Morrison and Stephenson<sup>4</sup> performed a weighted linear regression analysis of Mercury transit and eclipse measurements and concluded there had been no detectable trend in the solar semi-diameter since 1715. However, Gilliland<sup>8</sup> disputed this conclusion and claimed that the measurements supported a secular decrease. With the new data for 1981 and 1980, the whole sequence, covering 266 yr, is now re-examined to resolve this difference of interpretation.





**Fig. 1** Apparent changes in the semi-diameter of the Sun (arc s) between 1715 and 1981, deduced from timings of transits of Mercury (●) and total solar eclipses (○). The zero point corresponds to 959.63 arc s.

The previous regression analysis<sup>4</sup> weighted the individual means with their standard errors as this was felt to reflect the differences in numbers of reports for each event (which vary from 7 to 236) as well as the spread of reported timings. To assess the significance of a possible trend, the regression analysis has been repeated by weighting each mean with its standard deviation instead, except for the 1980 and 1981 eclipses where the ranges were used. This procedure reflects the actual spread of the data more completely and preserves the possible observer errors discussed earlier.

The secular trend in  $\delta R$  from the entire set of Mercury transits (2,150 timings) and solar eclipses (110 timings) is then given by

$$\delta R \text{ (arc s per century}^{-1}\text{)} \\ = -0.004 (\pm 0.193) + 0.023 (\pm 0.086) T \quad (1)$$

where  $T$  is the time from 1700 measured in centuries and the errors are  $\pm 1$  s.d.

This result seems to reduce dramatically the likelihood of a secular decrease, as the 'trend' computed above is nearly two orders of magnitude lower than that claimed by Eddy and Boornazian<sup>2</sup> and a factor of 4 less than that found previously by Parkinson *et al.*<sup>4</sup>. In fact, the slope in equation (1) is consistent with zero at the 80% level of significance. When different weighting functions are used, (for example, standard error, number of observations, or uniform weighting), the estimated gradients are changed, and even the sign is not preserved. The magnitude and sign of the trend obtained depend on the choice of weighting function used and cannot be inferred to result from a variation of the Sun. Thus there appears to be no evidence to support any secular trend in the solar diameter between 1715 and 1981.

In order to investigate possible biases between the Mercury transit and the solar eclipse timings, each data set has been examined separately. The best fitting regression lines are as follows:

Mercury transits:

$$\delta R \text{ (arc s per century}^{-1}\text{)} \\ = 0.172 (\pm 0.334) - 0.057 (\pm 0.202) T \quad (2)$$

total eclipses:

$$\delta R \text{ (arc s per century}^{-1}\text{)} \\ = -0.155 (\pm 0.278) + 0.077 (\pm 0.111) \quad (3)$$

These functions differ from zero by  $<0.1$  arc s for at least the past 150 yr, during which 87% of the measurements were made, and justify the combined analysis that led to equation (1).

Several solar semi-diameter measurements have been reported<sup>9</sup> from measurements of the width of the total eclipse zone on the ground. The recent measurements in 1976, 1979 and 1980 are in good agreement with the timing measurements, being essentially consistent with zero deviation. Their two historical measurements, however, warrant some comment. For

the 1715 eclipse, the location of the single observer at the northern limit is not known with certainty, and this may have led to an incorrect calculation of the semi-diameter. This is not the case for the 1925 eclipse where the location of the single observer at the northern limit was known with considerable accuracy and the southern limit was well determined. From the width of the total zone a value of  $+0.62$  arc s (standard error 0.08 arc s) was reported for  $\delta R$ . This must be compared with 11 timings of the same eclipse which gave  $\delta R = +0.18$  arc s (standard deviation 0.61 arc s) and 99 timings of the 1924 mercury transit which gave  $\delta R = +0.58$  arc s (standard deviation 1.06 arc s). It therefore appears that in 1924–25 the semi-diameter of the Sun was  $\sim 0.5$  arc s larger than its average value. However, it would be unjustified to claim that such an excursion was indicative of the Sun's secular behaviour. Indeed, the 80-yr periodicity deduced from the Mercury transit observations<sup>4</sup> reached a maximum in 1926. Thus the 1925 eclipse observations can be taken as support for the reality of this periodicity.

It is likely that changes in the size of the Sun will be accompanied by changes in its luminosity, the relative magnitudes and phases being characteristic of the perturbing mechanism. From the observations discussed here the largest change in semi-diameter would be associated with the 80-yr cycle. Consider, for example, the interval 1926–66, during which the average decrease in the solar semi-diameter,

$$\frac{\delta R}{R} = 0.0013 \text{ yr}^{-1}.$$

From a thorough analysis of the ground-based solar-constant monitoring programme of the Smithsonian Astrophysical Observatory, Hoyt<sup>10</sup> has concluded that there is no compelling evidence for a long-term trend between 1902 and 1962. Between 1923 and 1954, however, he found superficial evidence for an increase of  $0.17 \pm 0.04\%$  in the solar constant, but as he pointed out this may be an overestimate due to measurement and analysis errors. Thus it is likely that

$$0 \leq \frac{\delta L}{L} \leq 0.005\% \text{ yr}^{-1}$$

during this time.

If these changes are related, then following Sofia *et al.*<sup>11</sup>, a parameter,  $W$ , can be evaluated where

$$|W| = \left| \frac{\delta \log R}{\delta \log L} \right| \geq 0.26$$

Attempts to model variations resulting from changing the photospheric convection mixing length give  $W \sim +10^{-3}$  (ref. 12), so the value found above effectively rules out this as the sole process. Larger values of  $W$  can result from deeper-seated perturbations<sup>13</sup>. However, the theoretical models predict that the diameter and luminosity changes should be in phase, whereas here they appear to be out of phase.

The 80-yr period in the semi-diameter is reminiscent of a similar period reported in sunspot numbers. Several authors have discussed cyclic undulations in the peaks of successive 11-yr cycles, reporting periods in the range 55–90 yr, the exact values depending on the data span and the method of analysis used (see for example, ref. 14 and references therein). Such changes are most easily recognizable in the nineteenth century where a clear maximum appeared around 1850 and minima around 1810 and 1890. For comparison the cyclic function fitted to the semi-diameter reached a maximum in 1846 and minima in 1806 and 1886. Whilst this apparent phase agreement is not continued into the twentieth century, due perhaps to the erratic nature of successive 11-yr sunspot cycles, it does suggest that the semi-diameter variations and magnetic field changes may be related.

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1. Iben, I. *Astrophys. J.* **147**, 624–663 (1967).
2. Eddy, J. A. & Boornazian, A. A. *Bull. Am. astr. Soc.* **11**, 437 (1979).
3. Shapiro, I. I. *Science* **208**, 51–53 (1980).
4. Parkinson, J. H., Morrison, L. V. & Stephenson, F. R. *Nature* **288**, 548–551 (1980).
5. Parkinson, J. H. *Q. J. R. astr. Soc.* **23**, 252–255 (1982).
6. Duncombe, J. S. U. S. *Naval Observ. Circ.* No. 141 (1973).
7. Jones, J. E. *J. Br. astr. Ass.* **91**, 363–367 (1981).
8. Gilliland, R. L. *Astrophys. J.* **248**, 1144–1155 (1981).
9. Dunham, D. W., Dunham, J. B., Fiala, A. D. & Sofia, S. in *Variations of the Solar Constant* (ed. Sofia, S.) 117–121 (NASA CP-2191, Washington, 1981).
10. Hoyt, D. V. *Rev. Geophys. Space Phys.* **17**, 427–458 (1979).
11. Sofia, S., O'Keefe, J., Lesh, J. R. & Endal, A. S. *Science* **204**, 1306–1308 (1979).
12. Endal, A. S. & Twigg, L. W. *Astrophys. J.* **260**, 342–352 (1982).
13. Gough, D. O. in *Variations of the Solar Constant* (ed. Sofia, S.) 185–206 (NASA CP-2191, Washington, 1981).
14. Lomb, N. R. & Anderson, A. P. *Mon. Not. R. astr. Soc.* **190**, 723–732 (1980).

## Internal rotation of the Sun

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The low surface rotation rate of the Sun and other main-sequence stars is believed to be the result of angular momentum loss due to a stellar wind<sup>1</sup>. This loss also leads to a differential rotation, the interior spinning more rapidly than the surface. The rate of increase with depth of the rotation speed is limited by hydrodynamic instabilities, which cause an outward diffusion of angular momentum<sup>2</sup>. The conditions for the occurrence of hydrodynamic instabilities in a radiative stellar interior are reviewed here assuming that the rotation is constant on spheres. The instability with the lowest threshold is a double diffusive one, the axisymmetric baroclinic diffusive (ABCD) instability. A minimum rotation curve for the present Sun is calculated using the assumption that the efficiency of this instability is sufficiently high that the rotation of the Sun is close to marginal stability. This lower limit to the internal rotation rate is roughly consistent with present observations of the rotational splitting of solar oscillations.

In principle the rotation rate  $\Omega$  can be a function of both radius ( $r$ ) and latitude ( $\Lambda$ ). However, it has been conjectured<sup>3</sup> that if  $\Omega$  is not independent of  $\Lambda$ , finite amplitude shear instability will rapidly redistribute angular momentum along equipotential surfaces, so as to make  $\Omega$  constant on such surfaces. We assume here that this is the case, and that

$$\Omega \sim r^{-\alpha} \quad (1)$$

Mixing of angular momentum across equipotential surfaces can occur by four instabilities. The condition for finite amplitude shear instability across equipotential surfaces in the presence of both stable thermal and molecular weight stratification is, approximately (ref. 3, and J. P. Zahn, personal communication),

$$\alpha > \frac{N}{\Omega} \max \left[ \left( \frac{\nu}{\kappa} Re^c \right)^{1/2}, \left( \frac{H d \ln \mu / dr}{\nabla_a - \nabla} \right)^{1/2} \right] \quad (2)$$

where  $\mu$  is the mean molecular weight, produced by  $^1\text{H}$  and  $^4\text{He}$ ,  $\nabla$  is the logarithmic temperature gradient  $d \ln T / d \ln P$ ,  $\nabla_a$  is the adiabatic gradient,  $H$  is the pressure scale height and  $N$  the Brunt–Väisälä frequency. The molecular diffusivities of angular momentum, temperature and  $^4\text{He}$  are denoted by  $\nu$ ,  $\kappa$  and  $\kappa_4$ , respectively;  $Re^c$  is the critical Reynolds number for onset of turbulence ( $Re^c \approx 2,000$ ).

A sufficient condition for stability against the (adiabatic) baroclinic instability<sup>4</sup> for a rotation law of the form of equation

(1) is that everywhere in the star (H.C.S. and E.K., in preparation)

$$1 + 2 \sin^2 \Lambda \frac{r}{\rho} \frac{d}{dr} \left( \alpha \frac{\Omega^2}{N^2 \rho} \right) > 0 \quad (3)$$

where  $\rho(r)$  is the density. The quasigeostrophic approximation on a  $\beta$ -plane has been used in deriving this result. Since  $\Omega/N$  is small in a slowly rotating star, this condition is satisfied everywhere except near the centre of the star or near a convection zone, where  $N$  becomes small.

The condition for the existence of the Goldreich–Schubert–Fricke (GSF) instability, in the presence of a molecular weight gradient, is<sup>5</sup>

$$\frac{\nu}{\kappa} N_T^2 - \frac{\nu}{\kappa_4} N_\mu^2 < MN_\Omega^2 \quad (4)$$

where

$$N_T^2 = \frac{g}{H} (\nabla_a - \nabla) \quad (5)$$

$$N_\mu^2 = -g d \ln \mu / dr \quad (6)$$

$$N_\Omega^2 = \frac{1}{\omega^3} |\nabla(\tilde{\omega}^4 \Omega^2)| \quad (7)$$

$$M = \frac{\sin^2 \Gamma}{4 \sin \Lambda \sin (\Lambda - \Gamma)} \quad (8)$$

$$N^2 = N_T^2 + N_\mu^2 + N_\Omega^2 \approx N_T^2 \quad (9)$$

where  $\tilde{\omega}$  is the radial cylindrical coordinate and  $\Gamma$  the angle between surfaces of constant specific angular momentum  $\tilde{\omega}^2 \Omega$  and the rotation axis. For spherical rotation (equation (1)), the right-hand side of equation (4) becomes

$$MN_\Omega^2 = (\frac{1}{2} \Omega \alpha \cos \Lambda)^2 \quad (10)$$

Thus, it is easiest to satisfy equation (4) near the equator ( $\Lambda = 0$ ), and consequently GSF instability occurs when

$$\alpha \geq 2 \left( \frac{\nu}{\kappa} \right)^{1/2} \frac{N_T}{\Omega} \left( 1 - \frac{\kappa}{\kappa_4} \frac{H d \ln \mu / dr}{\nabla_a - \nabla} \right)^{1/2} \quad (11)$$

(We note that the limiting form of equation (4) for  $\Lambda \rightarrow 0$  is not the same as the instability condition at the equator. At the equator, instability is possible only for  $\Gamma = \pi$ , that is if the angular momentum decreases outward<sup>6</sup>.) Contrary to common belief, the GSF instability does not set in as soon as the rotation deviates from constancy on cylinders; owing to the effect of viscosity it requires a finite baroclinicity<sup>5–10</sup>. As the thermal diffusivity  $\kappa$  is much larger than  $\kappa_4$  ( $\kappa_4 \leq \nu$ ), then even a small gradient of molecular weight would have a strongly stabilizing effect on both the shear instability and the GSF instability.

In a differentially-rotating star one more instability (ABCD) can occur owing to the finite thermal diffusivity (refs 5, 8, and I.R., in preparation). The condition for its occurrence is

$$\frac{\nu}{\kappa} N_T^2 < \frac{1}{2} MN_\Omega^2 \quad (12)$$

For spherical rotation, this becomes

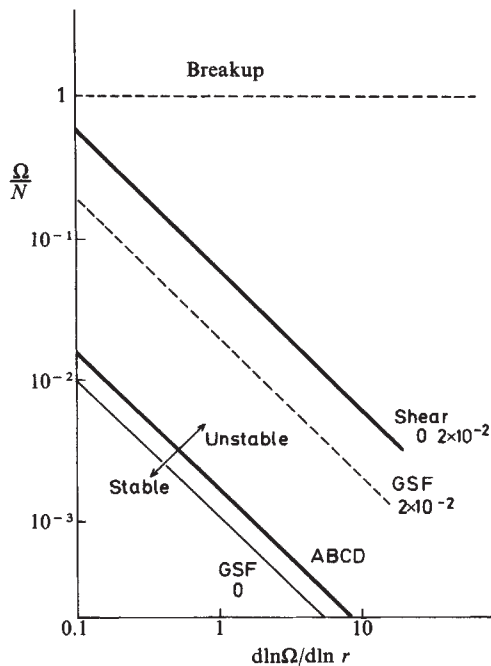
$$\alpha > 2\sqrt{2} \left( \frac{\nu}{\kappa} \right)^{1/2} \frac{N}{\Omega} \quad (13)$$

This result holds for all values of  $d \ln \mu / dr$  if the molecular weight is constant on constant-pressure surfaces. If  $\mu$  varies on such surfaces, the result still holds if  $H d \ln \mu / dr$  is small compared with unity.

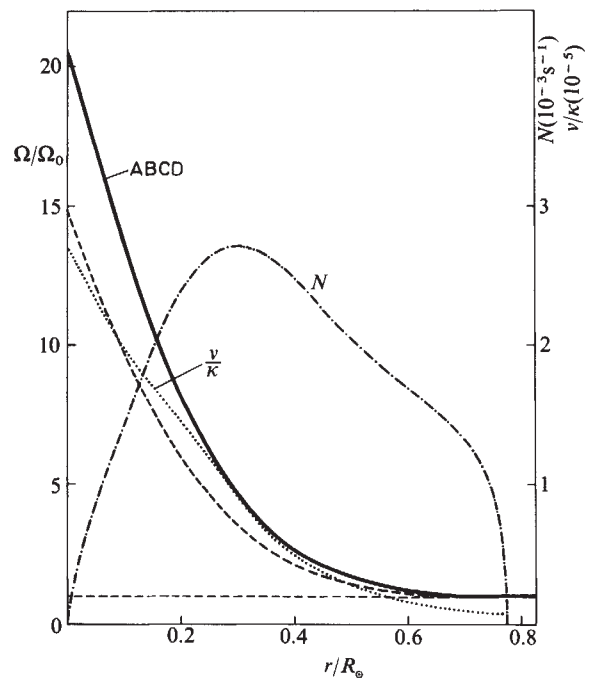
The factor  $N/\Omega$  appearing in all the conditions measures the ratio of gravitational to centrifugal forces. A radiative star is stable against breakup only if this ratio is  $\geq 1$ .

The instability conditions (equations (2), (3), (11) and (13)) are illustrated in Fig. 1 for the case  $\nu/\kappa = 10^{-6}$ , and for two values of  $d \ln \mu / dr$ . The most violent instability is the adiabatic baroclinic instability; it will cause a redistribution of angular





**Fig. 1** Lines of marginal stability for the shear, GSF and ABCD instabilities as functions of the rotation rate  $\Omega$  and its gradient.  $N$  is the Brunt-Väisälä frequency. Shear and GSF instability are given for  $N_\mu/N = 0$  and  $2 \times 10^{-2}$  (corresponding to molecular weight gradients  $-H d \ln \mu / dr = 0$  and  $\approx 4 \times 10^{-5}$  respectively).



**Fig. 2** Rotation rate  $\Omega$  in the solar interior (solid curve) in units of the surface rotation rate  $\Omega_0$ , derived from marginal ABCD instability. The dashed curve is the same for GSF instability (but with  $d\mu/dr = 0$  taken artificially). The Prandtl number  $Pr = \nu/\kappa$  and the Brunt-Väisälä frequency  $N$  are also shown.

momentum on a time scale of the order  $\Omega^{-1}$ . Equation (3) suggests that it occurs only in rather extreme conditions, however,  $\alpha \gg 1$  or  $N \approx \Omega$ . The finite amplitude shear instability has an intermediate position. The effective diffusivity due to this instability is of the order  $\kappa$  and the associated time scale  $\tau_d$  for angular momentum redistribution,  $\tau_d \sim r^2/\kappa$ , is  $\sim 10^7$  yr for the Sun. The GSF instability requires the smallest amount of differential rotation as long as  $H d \ln \mu / dr \ll \kappa_4/\kappa$ . Its effective diffusivity is hard to estimate but all recent estimates<sup>11-13</sup> suggest that it is very low. For the  $\mu$ -gradients that occur throughout most of a star the ABCD instability has by far the lowest threshold. Its effectiveness in diffusing angular momentum remains to be analysed, however.

On the basis of the above, we can now derive a 'minimum' rotation curve  $\Omega(r)$  for the present Sun, as follows. We assume that the instability with the lowest threshold (ABCD) is sufficiently effective in redistributing angular momentum that it keeps the angular momentum distribution close to the threshold at all times during the spindown of the star. The minimum rotation of the present Sun then follows from the equation for marginal stability, together with the observed surface rotation rate. For the ABCD instability, we find from equation (13):

$$\Omega(r) = \Omega_0 + 2\sqrt{2} \int_r^{r_c} \left(\frac{\nu}{\kappa}\right)^{1/2} \frac{N}{r} dr \quad (14)$$

where  $r_c$  is the radius of the lower boundary of the convection zone and  $\Omega_0 = \Omega(r_c)$ . We assume  $\Omega_0 = 2.83 \times 10^{-6} \text{ rad s}^{-1}$ , the observed rotation rate of the longest lived coronal structures<sup>14</sup>. These structures rotate almost rigidly and are assumed to be anchored deepest in the convection zone. The variation of  $\nu/\kappa$  and  $N$  with  $r$ , and the resulting profile are shown in Fig. 2. For comparison, the profile obtained from marginal GSF instability is shown under the (unrealistic) assumption that the stabilizing  $\mu$ -gradient can be ignored.

Information on the internal rotation is available from the quadrupole moment of the Sun and from rotational splitting of solar oscillations. Claverie *et al.*<sup>15</sup> have obtained a splitting of  $0.75 \mu\text{Hz}$  for the 5-min oscillations, and derived from this that the average rotation rate  $\bar{\Omega}$  is twice the surface value  $\Omega_0$ .

The appropriate average for high order  $p$ -mode oscillations is approximately<sup>16</sup>

$$\bar{\Omega} = \int \Omega/c \, dr / \int 1/c \, dr \quad (15)$$

where  $c$  is the adiabatic sound speed. For the profile of Fig. 2 this average is  $\bar{\Omega} = 2.45 \Omega_0 = 6.9 \times 10^{-6} \text{ rad s}^{-1}$  ( $= 1.10 \mu\text{Hz}$ ). In calculating this average, the convection zone has been assumed to rotate uniformly at the rate  $\Omega_0$ . Most of the contribution to  $\bar{\Omega}$  comes from the convection zone and the layers directly below it. The sharp peak near  $r=0$  contributes little. The synodic value of  $\bar{\Omega}$  is  $6.7 \times 10^{-6} \text{ rad s}^{-1}$ , so that the  $p$ -mode splitting would be  $1.07 \mu\text{Hz}$  (neglecting the influence of Coriolis effects on the splitting<sup>16</sup>). This value is in approximate agreement with the observed value<sup>15</sup>. From a preliminary analysis of the low-frequency oscillations measured by Hill, Gough<sup>17</sup> has derived possible solutions for  $\Omega(r)$ . The mode identifications on which these results rely are uncertain and have not yet been corroborated. If the results are taken at face value, however, they conflict with the rotation rates of Fig. 2, which are too high at the centre and too low in the outer layers. In our view, the overall observational evidence available is not strong enough to rule out the rotation curve of Fig. 2.

We stress that our profile represents the minimum allowed by the presently known hydrodynamic instabilities. If new determinations of the rotational splitting of solar oscillations would indicate lower internal rotation rates than that of Fig. 2 anywhere in the Sun, the ABCD instability would be ruled out as the angular momentum transport mechanism, as the condition for this instability is a local one. If one accepts our finding that the ABCD instability is the most readily occurring hydrodynamic instability, this would rule out such instabilities as the main mechanism of angular momentum transport in the Sun. Transport by the  $^3\text{He}$  instability<sup>18,19</sup>, by magnetic stresses or by magnetohydrodynamic instabilities would then have to be considered. If, on the other hand, higher rotation rates are found, the ABCD instability will occur, but one would still have to show that its effectiveness at transporting angular momentum is sufficiently high.

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1. Schatzman, E. *Ann. Astrophys.* **25**, 18–29 (1962).
2. Schatzman, E. *Astrophys. Lett.* **3**, 139–140 (1969).
3. Zan, J.-P. in *Stellar Stability and Evolution* (eds Ledoux, P., Noels, A. & Rogers, A. W.) 185 (Reidel, Dordrecht, 1974).
4. Hart, J. E. A. *Rev. Fluid Mech.* **11**, 147–172 (1979).
5. Knobloch, E. & Spruit, H. C. *Astr. Astrophys.* (submitted).
6. Knobloch, E. & Spruit, H. C. *Astr. Astrophys.* **113**, 261–268 (1982).
7. Roxburgh, I. W. in *Basic Mechanisms of Solar Activity* (eds Bumba, V. & Kleczek, J.) 453–466 (Reidel, Dordrecht, 1976).
8. McIntyre, M. E. *Geophys. Fluid Dyn.* **1**, 19–57 (1970).
9. Acheson, D. J. *Phil. Trans. R. Soc.* **289A**, 459–500 (1978).
10. Busse, F. H. & Chen, W.-L. *Geophys. Astrophys. Fluid Dyn.* **17**, 199–213 (1981).
11. James, R. A. & Kahn, F. D. *Astr. Astrophys.* **5**, 232–239 (1970).
12. Kippenhahn, R., Ruschenplatt, G. & Thomas, H.-C. *Astr. Astrophys.* **91**, 175–180 (1980).
13. Knobloch, E. *Geophys. Astrophys. Fluid Dyn.* **22**, 133 (1982).
14. Antonucci, E. & Doderio, M. A. *Solar Phys.* **62**, 107–122 (1979).
15. Claverie, A., Isaak, G. R., McLeod, C. P., van der Raay, H. B. & Roca Cortes, T. *Nature* **293**, 443–445 (1981).
16. Gough, D. O. *Nature* **298**, 350–354 (1982).
17. Gough, D. O. *Nature* **298**, 334–339 (1982).
18. Dilke, F. W. W. & Gough, D. O. *Nature* **240**, 262–264 (1972).
19. Christensen-Dalsgaard, J., Dilke, F. W. W. & Gough, D. O. *Mon. Not. R. astr. Soc.* **169**, 429–445 (1974).

## Solar radius change between 1925 and 1979

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By analysing numerous reports, from different locations, on the duration of totality of the solar eclipses on 24 January 1925, and on 26 February 1979, we have found that the solar radius at the earlier date was 0.5 arcs, or 375 km larger than at the later date. The correction to the standard solar radius found for each eclipse was different when different subsets of the observations were used (for example, edge of path of totality timings compared with central timings), suggesting the existence of systematic inaccuracies in our knowledge of the lunar figure. However, the differences between the corrections for both eclipses were very similar for all subsets considered, indicating that changes of the solar size may be reliably inferred despite the existence of the lunar figure errors, as long as the proper consideration is made of the distribution of the observations. We consider that these results are strong evidence in support of the occurrence of solar radius changes on shorter than evolutionary time scales.

Standard theory of stellar structure and evolution indicates that the Sun, as a whole, is currently in a very stable and quiescent state. Slow structural changes only occur as a significant fraction of the solar hydrogen is converted into helium within the nuclear furnace located at the very centre of the Sun, and in the case of the radius, for example, an increase at the level of 4% per  $10^9$  yr is predicted<sup>1</sup>. Obviously, if the standard model provided an accurate representation of the Sun, no radius change should be detectable within recorded human history.

However, recent investigations indicate that significant changes of the solar radius may have occurred within the past few centuries<sup>2–4</sup>. If these changes are real, our current standard model is in need of modifications. In fact the results remain controversial<sup>5,6</sup>, and thus far no unchallenged proof has been presented for the occurrence of the radius changes.

The present determination was made on the basis of timed observations of total solar eclipses, particularly those made near the edges of the path of totality<sup>7</sup>. Among the older eclipses, that which occurred in 1925 is the best observed because its path crossed the most heavily populated area of the United States, and also in part due to good luck. For the 1979 eclipse, observations were planned with the size determination in mind, so good accuracy is expected. Both eclipses were in the same Saros cycle, whereby every 18 yr eclipse geometries virtually repeat.

Radius determinations from observations made near the path edges are affected by timing errors less than observations made near the path centre (Fig. 1). The accuracy of this technique depends on (1) our ability to locate accurately the edges of the path of totality, and (2) our knowledge of the location and depth of the lunar features (valleys) where the Sun completely disappears (second eclipse contact) and first reappears (third contact)<sup>3,7</sup>. The positions of the critical observers (those at the extreme limits of the eclipses) can be identified on US Geological Survey maps and measured to an accuracy of better than 30 m which corresponds to an error in the solar radius of 0.03 arcs. For these eclipses, errors in our knowledge of the lunar topography were minimized as the contacts were observed to occur at the same lunar features. This combination of circumstances has made the 1925–79 eclipse pair the optimal benchmark to examine the difference in the solar radius at points separated by tens of years.

E. W. Brown led a large and successful campaign to obtain positional observations of the total solar eclipse of 24 January 1925, with the objective of testing his calculations. Before the eclipse, a detailed article was published<sup>8</sup> describing various eclipse-observing projects organized for the event, and giving instructions on methods for making accurate timings of the duration of totality, and absolute times of the contacts when possible. In addition, individuals and groups were encouraged to straddle the predicted edge of the path of totality, and simply report whether a total eclipse occurred or not, specifying the exact location. A year after the eclipse, Brown published a comprehensive analysis of the eclipse observations<sup>9</sup>. In this article he listed references where accurate times of the eclipse by professional and some advanced amateur astronomers were published, and he relied on these for his analysis. None of the sites (mostly observatories) involved were very close to the edges of the path of totality, and most were near the path centre. Cold weather ( $-20^\circ\text{C}$ ) affected chronometers at some sites, causing errors in the reported absolute times.

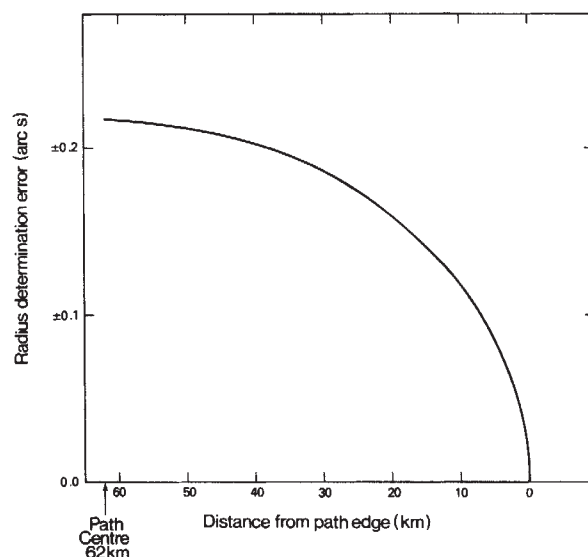


Fig. 1 Radius determination error produced by a timing error of 0.5 s as a function of distance from the edges of the path of totality for the 1925 eclipse.



Table 1 Timings made from the eclipse of 24 January 1925

| Event                                                                                                                                                              | Location on cresc. | No. beads | PA (deg) | WA (deg) | O - C (arcs) | Weight | Time corr. | Used in solution | Notes                  |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|-----------|----------|----------|--------------|--------|------------|------------------|------------------------|
| Central path observations from Fuertes Observatory, Cornell University, Ithaca, New York<br>(Long. = 5° 5' 56.5" W, Lat. = 42° 26' 51.4", elevation = 840 feet)    |                    |           |          |          |              |        |            |                  |                        |
| 14 h 8 min 45.10 s                                                                                                                                                 | Dis.               | Second    | 1        | 78.80    | 63.63        | 0.16   | 0.50       | 0.0 s            | Non-polar              |
| 14 10 37.10                                                                                                                                                        | Reap.              | Third     | 1        | 282.20   | 267.02       | 0.87   | 0.50       | 0.0              | Non-polar              |
| Central path observations from Yale University Observatory, New Haven, Connecticut<br>(Long. = 4° 51' 40.58" W, Lat. = 41° 19' 22.3", elevation = 131 feet)        |                    |           |          |          |              |        |            |                  |                        |
| 14 11 54.24                                                                                                                                                        | Dis.               | Second    | 1        | 107.00   | 91.83        | 0.56   | 0.46       | 0.24             | Non-polar              |
| 14 13 55.24                                                                                                                                                        | Reap.              | Third     | 1        | 280.99   | 265.81       | 0.35   | 0.46       | 0.24             | Non-polar              |
| 14 13 58.24                                                                                                                                                        | Merge              | Sev.      | 271.40   | 256.22   | 0.28         | 0.20   |            |                  | Not used               |
|                                                                                                                                                                    |                    |           | 286.19   | 271.01   | -0.30        | 0.05   | 0.24       |                  | Non-polar              |
|                                                                                                                                                                    |                    |           | 275.79   | 260.62   | 0.32         | 0.20   | 0.24       |                  | Non-polar              |
|                                                                                                                                                                    |                    |           | 287.59   | 272.41   | -0.41        | 0.05   | 0.24       |                  | Non-polar              |
| Central path observations from Vassar College Observatory<br>(Long. = 4° 55' 33.6" W, Lat. = 41° 41' 18", elevation = 200 feet)                                    |                    |           |          |          |              |        |            |                  |                        |
| 14 11 5.00                                                                                                                                                         | Dis.               | Second    | 1        | 94.00    | 78.83        | -0.43  | 1.00       | 0.0              | Non-polar              |
| 14 13 1.60                                                                                                                                                         | Reap.              | Third     | 1        | 282.19   | 267.02       | 0.53   | 1.00       | 0.0              | Non-polar              |
| Central path observations from Maria Mitchell Observatory, Nantucket<br>(Long. = 4° 40' 25.15" W, Lat. = 41° 16' 50", elevation = 98 feet)                         |                    |           |          |          |              |        |            |                  |                        |
| 14 16 9.20                                                                                                                                                         | Dis.               | Second    | 1        | 50.00    | 34.82        | 0.24   | 0.5        | 0.0              | Non-polar              |
| 14 17 38.20                                                                                                                                                        | Reap.              | Third     | 1        | 330.99   | 315.81       | -0.14  | 0.5        | 0.0              | Non-polar              |
| Central path observations from the Coast Guard cutter <i>Joutte</i> , docked in Rhode Island<br>(Long. = 71° 17' 21" W, Lat. = 41° 35' 17", elevation = 0 feet)    |                    |           |          |          |              |        |            |                  |                        |
| 14 14 55.59                                                                                                                                                        | Dis.               | Second    | 1        | 50.00    | 34.82        | -0.36  | 0.5        | 6.09             | Non-polar              |
| 14 16 7.59                                                                                                                                                         | Reap.              | Third     | 1        | 331.00   | 315.82       | -0.36  | 0.5        | 6.09             | Non-polar              |
| Central path observations from the East Chop lighthouse, Martha's Vineyard, Massachusetts<br>(Long. = 70° 34' 2.97" W, Lat. = 41° 28' 13.26", elevation = 40 feet) |                    |           |          |          |              |        |            |                  |                        |
| 14 15 50.64                                                                                                                                                        | Dis.               | Second    | 1        | 50.00    | 34.82        | 0.15   | 1.00       | 2.64             | Non-polar              |
| 14 17 1.84                                                                                                                                                         | Reap.              | Third     | 1        | 331.00   | 315.82       | 0.15   | 1.00       | 2.64             | Non-polar              |
| Central path observations from Mt Beacon, New York<br>(Long. = 73° 57' 12.8" W, Lat. = 41° 29' 17.6", elevation = 1,220 feet)                                      |                    |           |          |          |              |        |            |                  |                        |
| 14 10 44.78                                                                                                                                                        | Dis.               | Second    | 1        | 107.00   | 91.83        | -0.11  | 1.00       | 2.38             | Non-polar              |
| 14 12 40.58                                                                                                                                                        | Reap.              | Third     | 1        | 274.20   | 259.02       | -0.11  | 1.00       | 2.38             | Non-polar              |
| Central path observations from Guilford, Connecticut<br>(Long. = 72° 40' 57" W, Lat. = 41° 17' 9", elevation = 20 feet)                                            |                    |           |          |          |              |        |            |                  |                        |
| 14 12 11.19                                                                                                                                                        | Dis.               | Second    | 1        | 107.00   | 91.83        | 0.31   | 0.25       | 3.69             | Non-polar              |
| 14 14 12.19                                                                                                                                                        | Reap.              | Third     | 1        | 281.60   | 266.42       | 0.34   | 0.25       | 3.69             | Non-polar              |
| Northern limit observations from North Scituate, Rhode Island*<br>(Long. = 71° 35' 35" W, Lat. = 41° 50' 40", elevation = 352 feet)                                |                    |           |          |          |              |        |            |                  |                        |
| 14 15 } See note                                                                                                                                                   | Dis.               | Second    | 1        | 19.00    | 3.82         | -0.49  | 1.00       | —                | Polar                  |
| 14 15 }                                                                                                                                                            | Reap.              | Third     | 1        | 8.80     | 353.62       | -0.66  | 1.00       | —                | Polar, 2-, 3-parameter |
| Southern limit observations from 240 N. Riverside Drive, New York City<br>(Long. = 73° 58' 28.9", Lat. = 40° 47' 45.4", elevation = 130 feet)                      |                    |           |          |          |              |        |            |                  |                        |
| 14 14 } See note                                                                                                                                                   | Dis.               | Second    | 1        | 177.00   | 161.82       | -0.44  | 1.00       | —                | Polar, 2-, 3-parameter |
| 14 14 }                                                                                                                                                            | Reap.              | Third     | 1        | 195.80   | 180.62       | -0.53  | 1.00       | —                | Polar, 2-, 3-parameter |
| Southern limit observations from Leonia, New Jersey<br>(Long. = 73° 59' 19" W, Lat. = 40° 51' 50.5", elevation = 98 feet)                                          |                    |           |          |          |              |        |            |                  |                        |
| 14 10 37.10                                                                                                                                                        | Dis.               | Second    | 1        | 170.80   | 155.62       | 0.05   | 0.3        | 0.0              | Polar, 2-, 3-parameter |
| 14 11 21.00                                                                                                                                                        | Reap.              | Third     | 1        | 205.40   | 190.22       | -0.29  | 0.3        | 0.0              | Polar, 2-, 3-parameter |

The data are organized by observation station giving the identification and location for each observation station. The events reported are the disappearance (Dis.) or reappearance (Reap.) of a bead, the formation of a bead (crescent breaking up), and the merger of two or more beads (crescent forming). The location on the crescent is the description as reported, second or third contact, or south, north, centre, or left on the crescent as seen by the observer. The number of beads were as reported, with, in some cases, the observer only able to report that several or many were seen. The location determined by the reduction process for the timed event is given in position angle (PA) and Watts angle (WA) measured on the lunar limb. The O - C is the height of the solar limb above the predicted lunar limb from Watts charts. The weights assigned to the observations were determined from the initial residual analyses. The solution column indicates in which solutions presented in Table 3 the observations were used. Time corrections for the 1925 eclipse data, or personal equation (observer reaction time) are given in Table 1 or Table 2 when applicable. The times given in Tables 1 and 2 are those after corrections or personal equation have been applied. Some of the 1925 observational information has been published elsewhere. None of the 1979 observations have been published previously.

\* Observations by F. Seagrave.

Brown discussed briefly the observations made close to the edges of the eclipse path, and in particular the observations made by about 140 employees of the Affiliated Electric Companies of New York City. They bracketed the southern limit, which crossed Manhattan at 96th Street, by observing from rooftops at one-block intervals<sup>10</sup>. The addresses where the bracketing observations were made were given, and we have used them to measure the position accurately from a detailed topographic map. Brown states: "In no case was there a discordant observation. Their results have been checked up by other independent observations of the same character. It then appears that any point of the edge can be determined by

observations of this character . . . to an error of 0.03 arcs in the declination of the edge of the Moon. Of course, . . . this may be affected by an error of the order of 0.5 arcs owing to the inequalities of the Moon's limb". The last sentence emphasizes that Brown could not use the accurate edge observations due to the lack of good information about the profile of lunar mountains and valleys. He never published an analysis of the hundreds of edge observation reports he received, only stating that, from a preliminary analysis, the path was "a trifle narrower (in agreement with our analysis) and a trifle north" of the path which he had predicted. (We have searched for the reports which Brown received without success, and would be grateful

Table 2 Timings made from the eclipse of 26 February 1979

| Event                                                                      | Location on cresc. | No. beads | PA (deg) | WA (deg) | O-C (arc s) | Weight | Pers. eqn | Used in solution | Notes                         |
|----------------------------------------------------------------------------|--------------------|-----------|----------|----------|-------------|--------|-----------|------------------|-------------------------------|
| Southern limit observations from Meyer Township, North Dakota*             |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 99° 53' 24.5" W, Lat. = 48° 21' 18.9", elevation = 1,570 feet)    |                    |           |          |          |             |        |           |                  |                               |
| 16 h 40 min 44.50 s                                                        | Dis.               | South     | 1        | 86.20    | 62.87       | 0.80   | 0.25      | 0.3 s            | Non-polar                     |
| 16 40 45.80                                                                | Dis.               | South     | 1        | 87.40    | 64.07       | 1.00   | 0.25      | 0.3              | Non-polar                     |
| 16 40 56.90                                                                | Dis.               | South     | 1        | 94.40    | 71.07       | 0.85   | 1.00      | 0.3              | Non-polar                     |
| 16 41 3.70                                                                 | Dis.               | South     | 1        | 98.80    | 75.47       | 0.80   | 1.00      | 0.3              | Non-polar                     |
| 16 41 18.50                                                                | Dis.               | South     | 1        | 107.00   | 83.67       | 0.15   | 0.25      | 0.3              | Non-polar                     |
| 16 41 21.40                                                                | Dis.               | South     | 1        | 109.20   | 85.87       | -0.12  | 0.25      | 0.3              | Non-polar                     |
| 16 41 22.80                                                                | Dis.               | South     | 1        | 111.40   | 88.07       | 0.36   | 1.00      | 0.3              | Non-polar                     |
| 16 41 24.90                                                                | Dis.               | South     | 1        | 114.60   | 91.27       | 0.56   | 1.00      | 0.3              | Non-polar                     |
| 16 41 25.90                                                                | Dis.               | South     | 1        | 116.20   | 92.87       | 0.36   | 1.00      | 0.3              | Non-polar                     |
| 16 41 32.10                                                                | Dis.               | South     | 1        | 123.40   | 100.07      | 0.25   | 1.00      | 0.3              | Non-polar                     |
| 16 41 32.40                                                                | Dis.               | South     | 1        | 122.20   | 98.87       | 0.49   | 1.00      | 0.3              | Non-polar                     |
| 16 41 49.50                                                                | Form.              | North     | Sev.     | 152.60   | 129.27      | -0.03  | 0.25      | 0.5              | Non-polar                     |
|                                                                            |                    |           |          | 158.20   | 134.87      | -0.05  | 0.25      |                  | Polar                         |
|                                                                            |                    |           |          | 175.80   | 152.47      | -0.15  | 0.25      |                  | Polar                         |
|                                                                            |                    |           |          | 174.00   | 150.67      | -0.19  | 0.25      |                  | Polar                         |
|                                                                            |                    |           |          | 150.00   | 126.67      | -0.21  | 0.25      |                  | Non-polar                     |
| 16 41 53.90                                                                | Dis.               | Centre    | 1        | 159.00   | 135.67      | -0.13  | 0.25      | 0.3              | Polar                         |
| 16 42 21.50                                                                | Dis.               | Centre    | 1        | 177.00   | 153.67      | 0.37   | 0.25      | 0.3              | Polar, 2-, 3-parameter        |
| 16 42 24.00                                                                | Dis.               | Second    | 1        | 170.80   | 147.47      | 0.29   | 1.00      | 0.3              | Polar, 2-, 3-parameter        |
| 16 42 37.70                                                                | Reap.              | Third     | 1        | 195.80   | 172.47      | -0.40  | 1.00      | 0.5              | Polar, 2-, 3-parameter        |
| 16 42 46.00                                                                | Reap.              | North     | 2        | 205.40   | 182.07      | 0.26   | 0.25      | 0.5              | Polar, 2-, 3-parameter        |
| 16 42 48.90                                                                | Reap.              |           |          | 186.00   | 162.67      | -0.47  | 0.50      | 0.5              | Event, location not specified |
| 16 43 20.40                                                                | Merg.              | Left      | 1        | 193.20   | 169.87      | -0.20  | 1.00      | 0.3              | Polar                         |
| 16 43 40.30                                                                | Merg.              | Left      | 1        | 191.20   | 167.87      | -0.08  | 1.00      | 0.3              | Polar                         |
| Southern limit observations from Sawyer, North Dakota†                     |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 101° 3' 18.7" W, Lat. = 48° 5' 19.9", elevation = 1,545 feet)     |                    |           |          |          |             |        |           |                  |                               |
| 16 39 42.50                                                                | Dis.               | Centre    | Sev.     | 159.60   | 136.27      | -0.13  | 0.25      | 0.5              | Polar                         |
|                                                                            |                    |           |          | 159.00   | 135.67      | 0.27   | 0.25      |                  | Polar                         |
| 16 40 5.70                                                                 | Dis.               | Second    | 2        | 170.80   | 147.47      | -0.49  | 0.50      | 0.3              | Polar, 2-, 3-parameter        |
| Northern limit observations from Cle Elum, Washington                      |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 55' 57" W, Lat. = 47° 11' 27", elevation = 1,900 feet)       |                    |           |          |          |             |        |           |                  |                               |
| 16 18                                                                      | Dis.               | Second    | 1        | 8.80     | -14.15      | 0.10   | 1.00      |                  | Polar, 2-, 3-parameter        |
| 16 18                                                                      | Reap.              | Third     | 1        | 357.40   | 334.07      | 0.18   | 1.00      |                  | Polar                         |
| Southern limit observations from Billings, Montana‡                        |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 108° 32' 45.4" W, Lat. = 46° 4' 24.3", elevation = 3,535.5 feet)  |                    |           |          |          |             |        |           |                  |                               |
| 16 27 48.70                                                                | Dis.               | Second    | 1        | 162.80   | 139.47      | 0.59   | 0.5       | 0.3              | Polar                         |
| Northern limit observations from Moses Lake, Washington§                   |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 119° 26' 51.25" W, Lat. = 47° 15' 44.70", elevation = 3,840 feet) |                    |           |          |          |             |        |           |                  |                               |
| 16 19 25.70                                                                | Dis.               | Second    | 1        | 20.00    | -3.33       | 1.01   | 1.00      | 0.3              | Polar                         |
| 16 19 46.50                                                                | Reap.              | Third     | 1        | 357.40   | 334.07      | 0.15   | 1.00      | 0.5              | Polar                         |
| Northern limit observations from Zeneta, Saskatchewan                      |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 102° 4' 3.6" W, Lat. = 50° 43' 50.0", elevation = 1,730 feet)     |                    |           |          |          |             |        |           |                  |                               |
| 16 42 43.70                                                                | Dis.               | Second    | 1        | 15.20    | -8.13       | 0.69   | 1.00      | 0.3              | Polar                         |
| 16 see note                                                                | Reap.              | Third     | 1        | 11.40    | -11.93      | 0.40   | 1.00      |                  | Polar                         |
| Station 2 northern limit observations from Ellensburg, Washington          |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 39' 17" W, Lat. = 47° 05' 00", elevation = 2,080 feet)       |                    |           |          |          |             |        |           |                  |                               |
| 16 17 57.50                                                                | Dis.               | Second    | 1        | 32.40    | 9.07        | 0.09   | 0.25      | 0.3              | Non-polar                     |
| 16 19 0.50                                                                 | Reap.              | Third     | 1        | 330.60   | 307.27      | 0.78   | 0.25      | 0.5              | Non-polar                     |
| Station 3 northern limit observations from Ellensburg, Washington          |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 31' 43.4" W, Lat. = 47° 01' 46.3", elevation = 1,730 feet)   |                    |           |          |          |             |        |           |                  |                               |
| 16 17 52.80                                                                | Dis.               | Second    | 1        | 35.60    | 12.27       | 0.65   | 0.25      | 0.3              | Non-polar                     |
| 16 18 56.30                                                                | Reap.              | Third     | 1        | 330.60   | 307.27      | -0.51  | 0.25      | 0.5              | Non-polar                     |
| Station 5 northern limit observations from Ellensburg, Washington          |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 29' 47.5" W, Lat. = 47° 2' 12.5", elevation = 1,816 feet)    |                    |           |          |          |             |        |           |                  |                               |
| 16 17 54.80                                                                | Dis.               | Second    | 1        | 35.60    | 12.27       | 0.85   | 0.25      | 0.3              | Non-polar                     |
| 16 19 0.60                                                                 | Reap.              | Third     | 1        | 330.60   | 307.27      | 0.23   | 0.25      | 0.5              | Non-polar                     |
| Station 6 northern limit observations from Ellensburg, Washington          |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 29' 39" W, Lat. = 47° 7' 30", elevation = 2,680 feet)        |                    |           |          |          |             |        |           |                  |                               |
| 16 18 16.20                                                                | Dis.               | Second    | 1        | 32.40    | 9.07        | 0.30   | 0.25      | 0.3              | Non-polar                     |
| 16 19 2.00                                                                 | Reap.              | Third     | 1        | 347.80   | 324.47      | 0.44   | 0.25      | 0.5              | Polar                         |
| Station 9 northern limit observations from Ellensburg, Washington          |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 29' 47" W, Lat. = 47° 4' 24", elevation = 2,050 feet)        |                    |           |          |          |             |        |           |                  |                               |
| 16 18 4.70                                                                 | Dis.               | Second    | 1        | 32.40    | 9.07        | 0.35   | 0.25      | 0.3              | Non-polar                     |
| Station 10 northern limit observations from Ellensburg, Washington         |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 29' 45" W, Lat. = 47° 3' 31.5", elevation = 1,961 feet)      |                    |           |          |          |             |        |           |                  |                               |
| 16 18 2.30                                                                 | Dis.               | Second    | 1        | 32.40    | 9.07        | 0.20   | 0.25      | 0.3              | Non-polar                     |
| Station 11 northern limit observations from Ellensburg, Washington         |                    |           |          |          |             |        |           |                  |                               |
| (Long. = 120° 25' 4.8" W, Lat. = 46° 53' 2", elevation = 2,780 feet)       |                    |           |          |          |             |        |           |                  |                               |
| 16 17 34.00                                                                | Dis.               | Second    | 1        | 49.80    | 26.47       | 0.46   | 0.25      | 0.3              | Non-polar                     |
| 16 18 59.20                                                                | Reap.              | Third     | 1        | 330.60   | 307.27      | 0.04   | 0.25      | 0.5              | Non-polar                     |

Observations carried out by \* D. W. Dunham; † H. Povenmire; ‡ K. L. Strait and B. Bitts; § D. Calbrick; || J. Bruton.

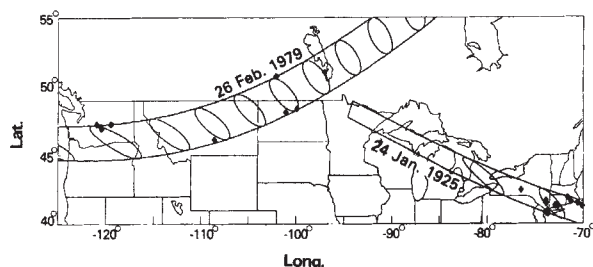


**Table 3** Corrections (in arc s) to the standard solar radius (959.63 arc s at a distance of 1 AU)

|                          | All data             | Polar                | Central<br>(non polar) | Polar solutions with same features |                      |
|--------------------------|----------------------|----------------------|------------------------|------------------------------------|----------------------|
|                          |                      |                      |                        | 3-Parameter                        | 2-Parameter          |
| <b>1925 eclipse</b>      |                      |                      |                        |                                    |                      |
| Correction (mean error)* | +0.21 ( $\pm 0.08$ ) | +0.48 ( $\pm 0.14$ ) | -0.02 ( $\pm 0.18$ )   | +0.39 ( $\pm 0.20$ )               | +0.49 ( $\pm 0.10$ ) |
| No. of timings           | 25                   | 6                    | 19                     | 5                                  | 5                    |
| Correlation coeff.†      | 0.49                 | 0.74                 | -0.71                  | 0.77                               | -0.23                |
| <b>1979 eclipse</b>      |                      |                      |                        |                                    |                      |
| Correction (mean error)* | -0.27 ( $\pm 0.06$ ) | -0.11 ( $\pm 0.09$ ) | -0.55 ( $\pm 0.07$ )   | -0.02 ( $\pm 0.22$ )               | +0.07 ( $\pm 0.12$ ) |
| No. of timings           | 47                   | 23                   | 23                     | 6                                  | 6                    |
| Correlation coeff.†      | -0.7                 | 0.09                 | -0.31                  | 0.77                               | -0.23                |
| <b>1979-25</b>           |                      |                      |                        |                                    |                      |
| Difference (mean error)  | -0.48 ( $\pm 0.10$ ) | -0.59 ( $\pm 0.17$ ) | -0.53 ( $\pm 0.19$ )   | -0.41 ( $\pm 0.30$ )               | -0.42 ( $\pm 0.16$ ) |

\* Our solutions for the correction to the standard solar radius.

† Correlation coefficients of the radius correction solution with either the relative ecliptic latitude or longitude correction, whichever had the largest absolute value.



**Fig. 2** Paths of totality for the solar eclipses of 1925 and 1979. The outline of the umbral shadow is shown at 6-min intervals. The 1925 eclipse began at sunrise in northern Minnesota. Observer locations are indicated by small diamonds. The map was prepared by F. Espenak.

for any data on the precise locations of any path-edge bracketing observations other than those which have been published.)

Fortunately, one observation made at the northern limit by astronomer Seagrave has been published<sup>11</sup>. A solar spectrograph was attached to the 8.5-inch equatorial refractor at Brown University's observatory at North Scituate, Rhode Island, and the slit was aligned tangent to the Sun's north limb. During the brief totality, every absorption line in the field of view became bright (emission). The 3 s of observed duration indicates, assuming circular geometry, that the observatory was only 17 m from the exact edge of the eclipse path.

A few timed observations of the eclipse were made from lighthouses and docked Coast Guard cutters in the path near the northern limit and were used in our analyses with reduced weight.

The observations from the 1925 eclipse used in our solutions are presented in Table 1. A constant offset of a few seconds had to be applied to the reported times of both contacts, preserving the reported duration of totality, for some of these observations due apparently to chronometer errors mentioned above. The constant offset was computed to equalize the radial residuals for the second and third contacts. These adjusted observations were included with reduced weight in the analyses.

The paths of both the 1925 and 1979 total eclipses and the locations of places where successful observations were made are shown in Fig. 2. The observational campaign to obtain timings of the 1979 eclipse from several locations just inside the edges of the path of totality was organized by the International Occultation Timing Association (IOTA) and has been briefly reported elsewhere<sup>3</sup>.

The observations used from the 1979 eclipse are reported in Table 2. This is similar to Table 1, except in this case, we applied no corrections to the times, as a consistent time standard was readily available to the observers. Personal equation (observer reaction times) were applied to the data.

The methods of data reduction are the same as those used previously<sup>12</sup>, but they are now more fully automated<sup>7</sup>, making practical more extensive analyses. Also, these results supercede some of our other published results because more data were

available, enough so that we can prepare solutions for polar and non-polar data, and also because some of the computed results for recent eclipses were previously presented with the wrong sign. The data sets for the 1925 and 1979 eclipses are large enough to form subsets for separate analyses, even after a few observations, which were clearly revealed to be discordant in the preliminary analysis, were removed.

Our solutions for the correction to the standard solar radius (959.63 arc s at a distance of 1 AU, or 149,597,871 km) are given in Table 3. We greatly reduced the effects of both systematic and random errors in Watts' lunar limb correction data<sup>13</sup> for some of the solutions by using only timings of events at the same lunar valley bottoms at the two eclipses. Because different numbers of timings, some with different weights, were made at the same feature during the two eclipses, the weights were adjusted so that the total weight for each feature was the same for each eclipse. Also, the total weights for the southern and northern limbs were made nearly equal. The usual solutions with this 'same feature' data set resulted in determinations with relatively poor accuracy and unacceptably large correlation coefficients. Both problems were satisfactorily eliminated by solving for only two parameters, corrections to the radius and latitude, leaving the longitude correction fixed at the value determined from the central data.

We consider the best solution to be the one in the last column of Table 3. The apparent depths of the lunar valleys more than about 30° from the lunar poles changed slightly between the two eclipses due to the different orientations (0.8° difference in longitude libration), contributing to the uncertainty of our central (non-polar) solution. The amount of error due to the change in depth for the features closer to the poles was less, because there was only a 0.1° difference in latitude libration. This solution for the change in the radius, 2.7 times its mean error, is in good agreement with the four mean error solutions given in the first two columns of Table 3, which also have low solution correlations. In fact, all solutions for the radius changes, even the one using only central data, are in good agreement. This gives us confidence in the results, and may even indicate that the systematic errors in Watts' charts are not as serious as we had expected for radius changes derived from the central eclipse data<sup>3</sup> for a sufficiently large number of observations. However, this latter point should be studied further with other pairs of eclipses where there is enough observational data.

Although we have shown that the solar radius decreased by 0.5 arc s between 1925 and 1979, the solar size in 1925 was not significantly different from that in 1715<sup>3</sup>. Therefore we conclude that the solar radius changes are not secular (monotonic and uniform). This is consistent with the null results of the transits of Mercury<sup>5,6</sup> which were less accurate than the eclipse results. The Mercury transit data convincingly disproved the existence of large secular changes in the solar radius. Figure 3 of ref. 6 shows a 0.5 arc s decrease in the solar radius which occurred during the 1930s and persisted to 1973, in agreement with our results. This same figure also includes data from the 1925 eclipse, for which they have plotted a correction of

$\sim 0.25$  arcs, in agreement with our result when all data from 1925 are used.

The Brown University Observatory is still used by the Sky-scrapers, an organization of amateur astronomers, and its accurate position, scaled from the detailed US Geological Survey map of the area, has been provided by John Cardillo Jr. Jeannie Behnke discovered the unpublished reports of the lighthouses and docked Coast Guard cutters in the US Naval Observatory Archives.

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1. Endal, A. S. & Sofia, S. *Astrophys. J.* **243**, 625–640 (1981).
2. Sofia, S., O'Keefe, J., Lesh, J. R. & Endal, A. S. *Science* **204**, 1306–1308 (1979).
3. Dunham, D. W., Sofia, S., Fiala, A. D., Herald, D. & Muller, P. M. *Science* **210**, 1243–1245 (1980).
4. Gilliland, R. L. *Astrophys. J.* **248**, 1144–1155 (1981).
5. Shapiro, I. I. *Science* **208**, 51–53 (1980).
6. Parkinson, J. H., Morrison, L. V. & Stephenson, F. R. *Nature* **288**, 548–551 (1980).
7. Sofia, S., Dunham, D. W. & Fiala, A. D. in *Proc. Conf. Ancient Sun*, 147 (Lunar and Planetary Science Institute, Houston, Texas 1980).
8. Russell, H. N. *Scient. Am.* January, 13 (1925).
9. Brown, E. W. *Astr. J.* **37**, 9–19 (1926).
10. Magalhães, F. V. *et al. Trans. Illumin. Engng Soc.* **20**, 565–628 (1925).
11. Seagrave, F. E. *Popular Astr.* **33**, 279 (1925).
12. Herald, D. *Moon* **15**, 91–107 (1976).
13. Watts, C. B. *Astr. Pap. Am. Ephemer.* **17**, (1964).

## Diffusional behaviour of entangled star polymers

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The dynamic properties of concentrated solutions and melts of linear polymers are reasonably well understood in terms of the reptation concept, whereby molecules are constrained to curvilinear motion within 'tubes' formed by entanglements with their neighbours<sup>1–3</sup>. Understanding of dynamics of entangled nonlinear polymers, however, is still rather limited. Reptation in such cases is expected to be strongly suppressed, but there is little direct evidence for this, and conflicting models have been proposed<sup>4–6</sup>. We report here a critical study of the diffusion coefficient  $D$  of a series of model linear and three-arm star-branched polymers, diffusing in an entangled linear polymer melt matrix, designed to elucidate dynamic mechanisms for the entangled stars. Measuring  $D$  as a function of diffusant degree of polymerization  $N$  we find, for the linear molecules,  $D \propto N^{-2}$ , as expected for purely reptative motion<sup>1,7–9</sup>. For the case of moderately short stars, however,  $D$  falls considerably faster than an inverse square power law, and is well fitted by an exponential type relation  $D \propto e^{-\alpha N}$ . There is evidence that for the longest stars used in the present study it is the release of entanglements by reptation of the linear matrix molecules which dominates the dynamics. This is the first direct indication of such 'tube-renewal' effects in a system of entangled polymers<sup>9</sup>.

The experimental technique and sample-preparation procedure have been described previously<sup>9,10</sup>; the approach involves monitoring, by IR microdensitometry, the diffusion broadening of an originally step-like concentration profile, of the deuterium labelled diffusant molecules, within the polymer matrix. The matrix used is a highly entangled linear polyethylene (HDPE1); the linear and three-arm star-branched polyethylene-like diffusant molecules were prepared by deuteration of the corresponding monodispersed 1,4-polybutadiene polymers<sup>11</sup>. Molecular characteristics are given in Table 1.

Figure 1 shows  $D(N)$  as a function of the degree of polymerization  $N$  of diffusants, in an HDPE1 melt at 176 °C. For the linear polymer a best-fit power relation (solid line,  $a$ )

$$D(N) = D_0 N^{-1.95 \pm 0.1}$$

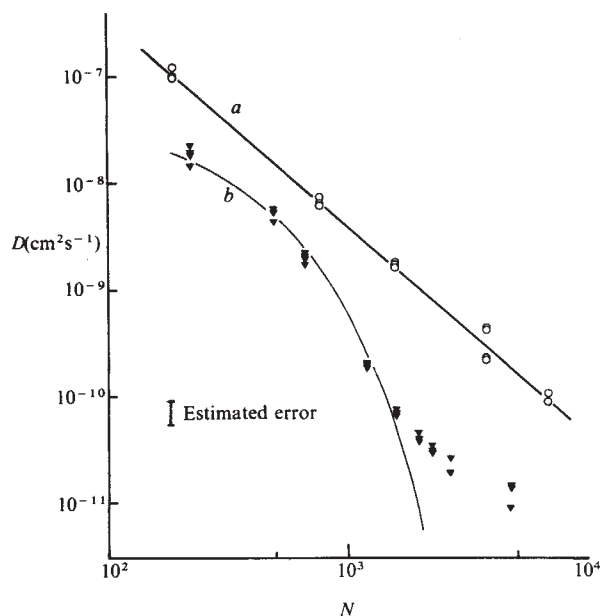
is indicated. This is in good agreement with the inverse square law ( $D \propto N^{-2}$ ) expected for pure reptation, and also compares well with the results obtained previously<sup>9</sup> for deuterated polyethylene diffusing in the same conditions. For the three-arm star polymers the variation of  $D$  with  $N$  is initially considerably more rapid than a power law. A good (least squares) fit to the first five points (17 experiments) is obtained by the exponential relation

$$D(N) = D_s e^{-\alpha N} \quad (1)$$

(curve  $b$ , Fig. 1).

This exponential suppression of  $D$  is consistent with the mechanism proposed by de Gennes<sup>4</sup>, whereby the reptation of the star-branched polymer entangled in a fixed network is hindered by the branching point. In order that translational diffusion of the star occurs, it is necessary that some retraction (rather than reptation) of an arm down its own 'tube' towards the centre monomer takes place; this leads to a relation of the type  $D \sim F(N) e^{-\alpha N}$ , where  $F(N)$  is a power function of  $N$  (typically  $N^{-2}$ ). Such a power-exponential relation is also consistent with the data. The crucial point, however, is the essentially exponential decay of  $D$  with  $N$  for the stars, for  $N \leq 1,500$ .

For the longer stars ( $N \geq 1,500$ ) we note a strong deviation from the exponential relation (curve  $b$ , Fig. 1). This may be attributed to the fact that the linear melt matrix is not strictly



**Fig. 1** The variation of  $D$  with degree of polymerization  $N$  for linear (○) and three-arm star-branched (▼) deuterated polybutadiene molecules diffusing in a linear polyethylene melt (HDPE1,  $M_w = 1.6 \times 10^5$ ,  $M_w/M_n \approx 15$ ) at  $176 \pm 0.5$  °C. Each point represents a separate experiment run over differing lengths of time (varying by a factor of up to  $\sim 4$  within sets of points), and is the mean of  $\sim 10$  independent concentration profile measurements<sup>9</sup>. Curve  $a$  is a regression analysis best-fit ( $r^2 = 0.99$ ) to the data for the linear diffusants (15 experiments),  $D = D_0 N^{-1.95 \pm 0.1}$ , where  $D_0 = (2.8 \pm 0.4) \times 10^{-3} \text{ cm}^2 \text{ s}^{-1}$ . Curve  $b$  is the best exponential fit (regression analysis,  $r^2 = 0.99$ ) to the data for the five shortest stars (17 experiments),  $D = D_s e^{-\alpha N}$ , where  $D_s = (4 \pm 1) \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ ,  $\alpha = 4.2 \times 10^{-3}$ .



**Table 1** Molecular characteristics of linear and three-arm star-branched polybutadiene (PBD) samples

|                         | Sample  | ( $M_w/10^3$ ) | ( $M_w/M_n$ ) | N     |
|-------------------------|---------|----------------|---------------|-------|
| Linear                  | CDS-B-3 | 2.6            | 1.04          | 190   |
|                         | LF-1    | 10.8           | 1.02          | 770   |
|                         | LF-2    | 22             | 1.04          | 1,570 |
|                         | LF-3    | 53             | 1.03          | 3,750 |
|                         | LF-4    | 96             | 1.06          | 6,830 |
| Three-arm star-branched | JK-8A   | 3.1            | 1.07          | 220   |
|                         | JK-5A   | 6.8            | 1.07          | 490   |
|                         | JK-6A   | 9.3            | 1.04          | 660   |
|                         | WG-2A   | 16.8           | 1.04          | 1,200 |
|                         | WG-1A   | 21.9           | 1.06          | 1,560 |
|                         | JK-4A   | 27.6           | 1.04          | 1,960 |
|                         | JK-3A   | 31.2           | 1.04          | 2,220 |
|                         | JK-2A   | 37.2           | 1.05          | 2,650 |
|                         | JK-1A   | 66.3           | 1.05          | 4,720 |

The molecular weights of the precursor PBD polymers (92% 1,4-PBD; 8% 1,2-PBD) were determined using osmometry and size-exclusion chromatography. The PBD samples, monomer structure  $-(CH_2-CH=CH-CH_2)-$ , were deuterated at high pressures<sup>11</sup> (to yield the polyethylene-like monomer structure  $-(CH_2-CHD-CHD-CH_2)-$ ) and then used as the diffusant molecules in the present study. The  $N$  values given are the numbers of main-chain  $CH_2/CHD$  units in the diffusants.  $M_w$  and polydispersities for the stars are obtained from the corresponding values for the linear precursors; independent measurements on the stars indicated a degree of branching  $f = 3.0 \pm 0.1$ . Control measurements following deuteration showed essentially complete saturation of the PBD, and negligible degradation.

speaking a fixed network. When  $D$  for the stars becomes sufficiently low, the motion of the linear melt molecules themselves leads to a relaxation of entanglements about the star polymers; this results in a more rapid diffusive motion than the exponentially slow arm-retraction mechanism. Such 'constraint-relaxation' or 'tube-renewal' effects are a natural consequence of the movement of the linear melt molecules<sup>8</sup>, and have been predicted<sup>12</sup>, but not hitherto observed, for entangled systems where reptation is suppressed.

The present study shows that, for three-arm star-branched molecules diffusing in a highly entangled linear matrix, the diffusion coefficient initially decreases exponentially with star molecular weight. This provides direct support for the notion that reptation of such stars in a fixed network is suppressed, and suggests that translational diffusion (and hence the longest relaxations) must take place by retraction of the star-arms within their 'tubes' of entanglements. For sufficiently long star molecules diffusing in the linear melt matrix, the results indicate that such a retraction mechanism gives way to one dominated by constraint release, due to the motion of the matrix molecules themselves.

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- de Gennes, P. G. *J. chem. Phys.* **55**, 572-579 (1971).
- Doi, M. & Edwards, S. F. *JCS Faraday II* **74**, 1789-1801 (1978).
- Ferry, J. D. *Viscoelastic Properties of Polymers* 3rd edn (Wiley, London, 1980).
- de Gennes, P. G. *J. Phys.* **36**, 1199-1207 (1975).
- Doi, M. & Kuzuu, N. *J. polym. Sci. Polym. Lett.* **18**, 775-780 (1980).
- Evans, K. *JCS Faraday II* **77**, 2385-2399 (1981); *J. polym. Sci. Polym. Lett.* **20**, 103-108 (1982).
- Kimmich, R. & Bachus, R. *Colloid Polym. Sci.* **260**, 911-936 (1982).
- Klein, J. *Macromolecules* **11**, 852-858 (1978).
- Klein, J. & Briscoe, B. J. *Proc. R. Soc. A* **365**, 53-71 (1979).
- Bauer, B. J. & Fetters, L. J. *Rubber Rev.* **51**, 406-465 (1978).
- Rachapudy, H., Smith, G. G., Raju, V. R. & Graessley, W. W. *J. polym. Sci. Phys.* **17**, 1211-1222 (1979).
- Klein, J. *Am. chem. soc. Polym. Reprints* **22**, 105-106 (1981); *Phil. Mag.* **A43**, 771-778 (1981).

## Mixing basaltic and dacitic magmas by forced convection

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Although magma mixing is an old concept which has been applied to many magma types, recent petrographic studies have revived interest in the theory by suggesting that it may account for the origin of calc-alkaline magma<sup>1-6</sup> and mid-ocean ridge basalt<sup>7-9</sup>. There has been no previous experimental investigation into how two magmas with different properties can mix to form a homogeneous magma of andesitic composition or to produce textures such as observed in banded dacite. We present here the results of experiments demonstrating that basaltic and dacitic magmas can be easily mixed by forced convection to form both banded dacite and homogeneous andesite in less than a few hours.

An olivine tholeiite, 1921 lava of Kilauea<sup>10</sup> and a dacite, of Mori-yoshi volcano, northern Japan<sup>11</sup>, were powdered then sintered to prepare two rods of separate compositions. The sintered rods were fixed to the upper and the lower shafts and melted in an IR radiation image convergence furnace<sup>12</sup>. When the ends of both rods were melted (around 1,250 °C), both were connected and rotated in opposite directions so that the two melts were mixed by forced convection. The run products were investigated under a polarizing microscope and chemically analysed using an energy-dispersive type electron probe micro-analyser, based on the method of Fujimaki and Aoki<sup>13</sup>. A series of experiments were performed by changing the duration of the run, rotation rate and the positions of the two rods.

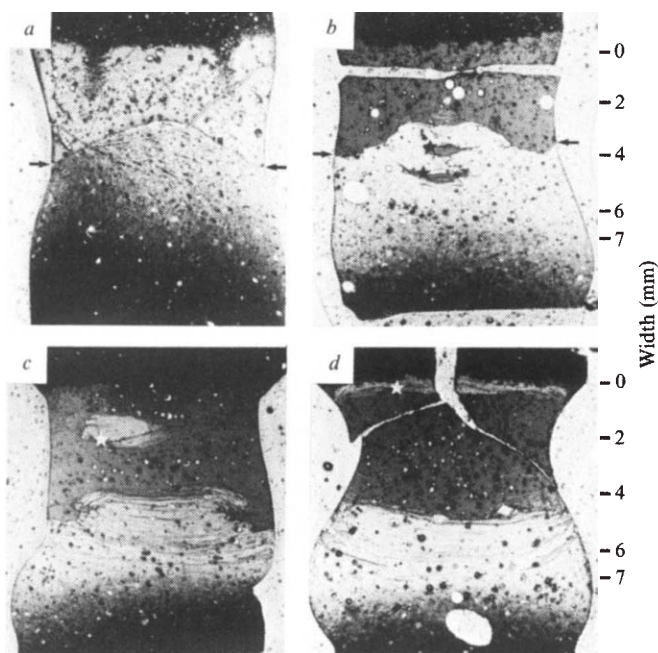
Figures 1 and 2 show photography and chemistry, respectively, of a series of products obtained with a constant rotation rate (48 r.p.m.) but different run durations. It is apparent that basaltic and dacitic magmas are easily mixed in <2 h to form: (1) an homogeneous magma of andesitic composition in the basaltic part and (2) a banded texture in the dacitic part. The texture and chemical composition formed by mixing were found to vary systematically as a function of run duration. Similar results were obtained when rotation rates were changed with a duration of the run kept constant. When the positions of the upper and lower rods were reversed, essentially similar results were obtained except for a reverse of the positions of homogeneous magma and banded texture.

Basaltic drops are trapped in the dacite melt to form streaks, whereas dacite drops are torn and rise into the basalt melt (Fig. 1). After 2 h, the melt in the basalt side becomes almost homogeneous, whereas in the dacite side, a banded texture develops, which has a similar appearance to naturally observed banded pumice<sup>14</sup>.

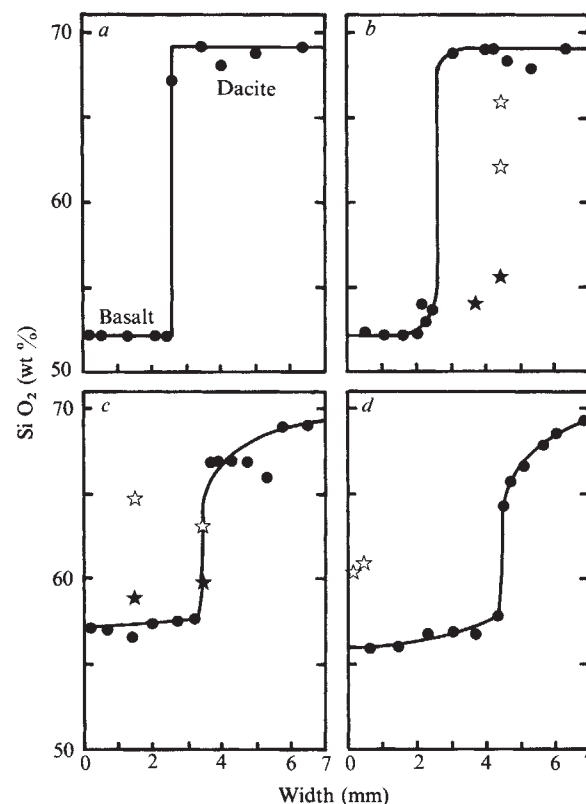
No change in composition is noted after 5 min (Fig. 2a), but after 1 h (Fig. 2b) the composition near the interface between the two melts starts to change. After 2 h (Fig. 2c), the basalt has changed completely to an andesitic composition ( $SiO_2 = 56\%$ ) even near the unmelted part. The compositions of the drops trapped in the melts of the opposite sides show a more drastic change than does the composition of the bulks. The volume of dacite melt decreases whereas that of andesite melt increases as the run duration or rotation rate increases.

The observations can be summarized as follows: (1) the chemical composition of basalt changes significantly to andesite as mixing proceeds, whereas that of the dacite melt remains unchanged, (2) the gradient of compositional change away from the interface with the unmelted part is rather gentle on the

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**Fig. 1** Transmission photomicrographs of the products obtained by a series of experiments with a constant rotation rate (48 r.p.m.) but different durations. *a*, 5 min; *b*, 60 min; *c*, 120 min; *d*, 200 min. Upper part, above 0 mm, is unmelted basalt, and the part lower than around 7 mm is unmelted dacite. From 0 to ~7 mm is a melted and mixed zone, where electron-probe microanalyses were made. Arrows indicate the interface between basalt and dacite melts. ☆, Dacite drops in basalt melt; ★, basalt drops trapped in dacite melts.



**Fig. 2** Plots of analytical data obtained for the products shown in Fig. 1. *a*, *b*, *c* and *d* are the same as in Fig. 1. ●, The data obtained for the bulk glass. ☆, Dacite drops trapped in basalt; ★, basalt drops in dacite melt.

basalt side compared with the much sharper gradient on the dacite side, and (3) drops trapped in the two melts show more a rapid compositional change than does the bulk.

As expected from a mixing process, the plot of the major components against the  $\text{SiO}_2$  of glasses for these samples shows linear relationships<sup>15</sup>.

We conclude that basalt and dacite melts with different viscosities, melting temperatures and diffusion coefficients can be easily mixed in <2 h to form an homogeneous andesite melt and to produce banded textures in the dacite, when forced convection is applied. Because the starting materials contained less volatile components than do natural magmas, mixing should have been more difficult in the experimental system, but a good mixture was obtained between the two magmas. Note here that the temperatures of the experimental system are rather high compared with the natural systems.

Forced convection is a more effective way of mixing the two magmas than is a static diffusion process. Yoder<sup>16</sup> and Watson<sup>17</sup> carried out diffusion experiments between basaltic and rhyolitic melts. The latter showed that the width of the mixed zone by diffusion was only 0.5 mm after 14 h at a pressure of 6 kbar and temperature of 1,200 °C, or after 6 h when the temperature was raised to 1,300 °C. Once bands having a width <0.5 mm appear during forced convection, mixing can proceed easily even if forced convection is stopped. Mixing can, of course, proceed more easily if forced convection is continued, since the streaks become thinner.

Compositional and textural changes of dacitic and basaltic drops trapped in the melts of the opposite sides (see Figs 1 and 2) represent the cases in which two melts with different volumes are mixed. Mixing only a small volume of dacitic magma having higher viscosity with a large volume of basaltic magma having a lower viscosity produced a rapid compositional change of the latter. When, on the other hand, a small volume of basalt is mixed with a large volume of dacite, this produced the banded appearance. In differentiating between the two cases, it may

be that the viscosity difference between the two magmas had the essential role, with a subsidiary effect of temperature difference, which produces the phase change<sup>18</sup>, and this may mean that the real situation differs from the experimental.

The composition of basaltic magma changes rather drastically by mixing with the dacitic magma, whereas that of dacite does not show such drastic change (compare the compositions of both sides near the unmelted ends in Fig. 2). By mixing, basalt changes easily to andesite, whereas dacite does not show such a rapid transformation. This implies that when eruption takes place during magma mixing, we will expect to find dacite and andesite but not the original basalt. Experimental details of this study will be published elsewhere<sup>15</sup>.

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1. Eichelberger, J. C. *Bull. geol. Soc. Am.* **86**, 1381–1391 (1975).
2. Anderson, A. T. *J. Volcanol. geotherm. Res.* **1**, 3–33 (1976).
3. Eichelberger, J. C. *Nature* **275**, 21–27 (1978).
4. Sakuyama, M. *J. Volcanol. geotherm. Res.* **5**, 179–208 (1979).
5. Luhr, J. F. & Carmichael, I. S. E. *Contr. Miner. Petrol.* **71**, 343–372 (1980).
6. Sakuyama, M. *J. Petrol.* **22**, 553–583 (1981).
7. Donaldson, C. H. & Brown, R. W. *Earth planet. Sci. Lett.* **37**, 81–89 (1977).
8. Dungan, M. A. & Rhodes, J. M. *Contr. Miner. Petrol.* **67**, 417–431 (1978).
9. Walker, D., Shibata, T. & DeLong, S. F. *Contr. Miner. Petrol.* **70**, 111–125 (1979).
10. Yoder, H. S. Jr & Tilley, C. E. *J. Petrol.* **3**, 342–532 (1962).
11. Nakagawa, M. *J. Jap. Ass. Miner. Petrol. Econ. Geol.* (in the press).
12. Akashi, T., Matsumi, K., Okada, T. & Mizutani, T. *IEEE Trans. Magn.* **MAG-5**, 285–289 (1969).
13. Fujimaki, H. & Aoki, K. *Sci. Rep. Tohoku Univ. Ser. III* **14**, 261–268 (1980).
14. MacDonald, G. A. & Katsura, T. *Bull. geol. Soc. Am.* **76**, 475–482 (1965).
15. Kouchi, A. & Sunagawa, I. *Sci. Rep. Tohoku Univ. Ser. III* **15**, 163–175 (1982).
16. Yoder, H. S. Jr *Am. Miner.* **58**, 153–171 (1973).
17. Watson, E. B. *Contr. Miner. Petrol.* **80**, 73–87 (1982).
18. Eichelberger, J. C. *Nature* **288**, 446–450 (1980).



## Perceptual organization in moving patterns

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Is human motion perception based on a local piecemeal analysis of the image or do global effects play an important role? We used metastable apparent motion displays (Fig. 2) to try to answer this question. Two spots were flashed simultaneously on diagonally opposite corners of a square and then switched off and replaced by two spots appearing on the remaining corners. One could either see vertical or horizontal oscillation of the spots and the display was bistable just as a Necker cube is. We found that if several such bistable figures were randomly scattered on the screen (Fig. 3*b*), and presented simultaneously, then one always saw the same motion-axis in all of them, suggesting the presence of global field-like effects for resolving ambiguity in apparent motion. Surprisingly, the appearance of these displays could not be influenced by voluntary effort unless the speed of alternation was very slow. (Less than 3 frames per second.) It may be that if the events in the module that computes apparent motion are too rapid then it cannot be coupled with the "will" mechanism, which may have a long time constant.

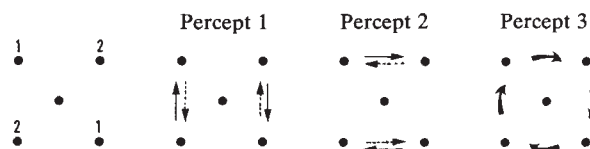
Metastability is one of the most striking yet enigmatic aspects of perception<sup>1-3</sup>. Necker cubes (Fig. 1) and other bistable figures are often used to illustrate the point that perception is really a hypothesis on the state of affairs in the world rather than a passive response to sensory stimuli. One's perception of the cube changes dramatically as the mind hesitates between alternative three-dimensional representations. Indeed, when viewing such bistable figures it is often hard to believe that something has not changed physically in the stimulus.

This report is concerned with metastable apparent motion displays such as Fig. 2; a matrix of four dots forming the corners of a square. It was generated on a P-4 phosphor cathode ray tube (CRT) using an Apple 2 microcomputer and viewed from a distance of 1 m. The sides of the square subtended 40 min of arc and the dots themselves were 3 min of arc in diameter. A hand-held potentiometer could be used to vary the speed of alternation or stimulus onset asynchrony (SOA) continuously over a wide range: the SOA was varied by changing the duration of each of the two frames and there was no dark interval (ISI) interposed between the frames. The number by each dot refers to the time at which it is presented. Dots on two diagonally opposite corners are flashed first and then switched off, followed by two dots appearing simultaneously on the remaining two corners, with the procedure repeating in a continuous cycle. The two possible percepts, are indicated in the diagram as Percept 1 (vertical oscillation) and Percept 2 (horizontal oscillation) and the display is essentially bistable just as a Necker cube is<sup>6</sup>. However, we found it almost impossible to voluntarily switch from one percept to the other when the speed of alternation was higher than 3 frames per second (SOA = 350 ms). Subjects almost always experience hysteresis, that is, they tend to persist in seeing one of the percepts (either vertical or horizontal oscillation) and can switch the axis of motion only by looking away and looking back after some time has elapsed.

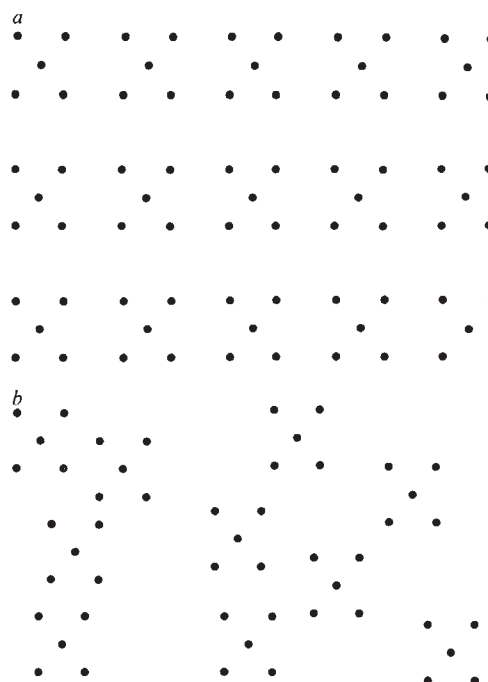
Another theoretically possible percept is either continuous clockwise (Percept 3) or anticlockwise motion but at short SOAs (350–400 ms), this was impossible to achieve even through intense mental effort. However, we made the curious observa-



**Fig. 1** An outline drawing of a transparent cube. The mind hesitates between the two alternative three-dimensional representations.

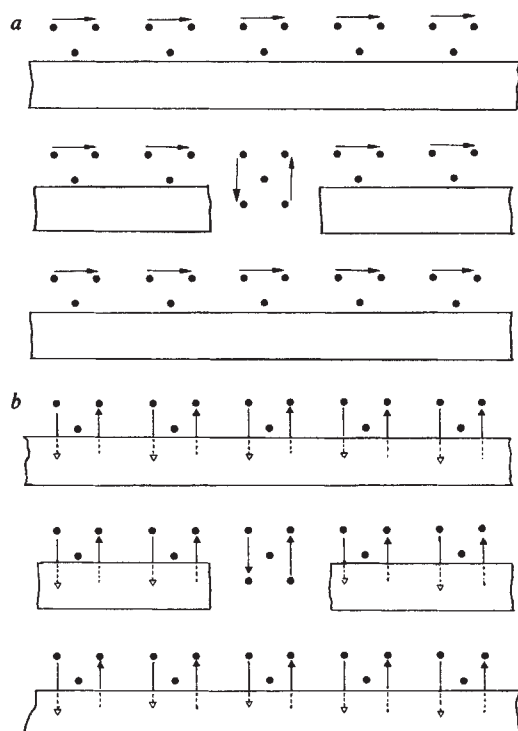


**Fig. 2** The basic bistable motion display used in our experiment. (Numerals indicate order of presentation.) The three possible percepts are shown. (A fourth percept, anticlockwise motion, is not shown.)



**Fig. 3** *a*, Multiple bistable displays presented simultaneously. Identical oscillations are observed in *all* the displays. *b*, The squares (bistable dot-pairs) are randomly positioned and the same effect is seen showing that the presence of regular arrays is not necessary. The central dot within each display is present continuously and is perceived to be static.

tion that when the SOA was made sufficiently long (>465 ms) subjects could occasionally will themselves to switch the motion axis or even to see clockwise or anticlockwise motion. Six naive subjects were asked to view the display and we then varied SOA over a wide range using a hand-held potentiometer. We began with very slow speeds so that the subject could will clockwise (or anticlockwise) motion and then gradually increased the speed of alternation. When the SOA was smaller than 465 ms, subjects reported that they could no longer hold this percept (mean of 6 readings  $\times$  6 subjects, that is, 36 readings = 465 ms; s.d. = 33). They could now only see oscillation, and the axis of oscillation could no longer be influenced at will. Furthermore, we find that if Fig. 2 and its mirror-image are presented side-by-side then at slow speeds one can simultaneously 'will' clockwise and anticlockwise motion suggesting that eye movements cannot explain the effect. We conclude that axis-dominance remains absolute and unambiguous at small SOAs and that it can be coupled with the effects of will and attention only at SOAs longer than 465 ms. Hence using

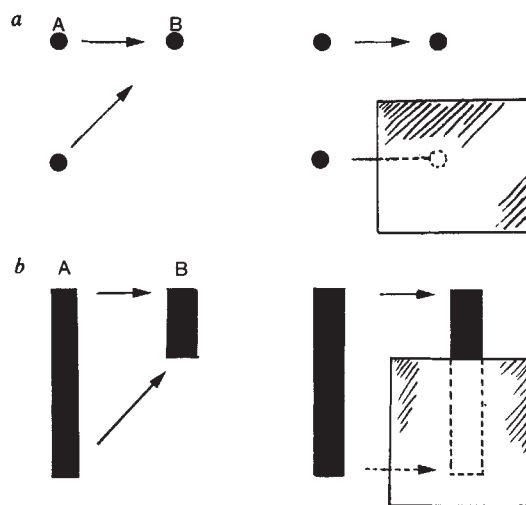


**Fig. 4** *a*, The effect of unambiguous motion in the surround on a single bistable display in the centre. This was produced by mounting strips of adhesive tape on Fig. 3*a*. *b*, 'Occult apparent motion' seen behind the masking tape. The effect disappears if the central inducing figure is removed. This suggests that the presence of motion in one region of the visual field somehow creates a template that allows one to imagine similar motion in other regions.

short SOAs may be a good way to study metastability without worrying about contamination from higher centres.

We have now begun to use our bistable displays to address the more general question of whether motion perception is based on a piecemeal analysis of the image or whether global field-like effects are also important as emphasized by Gestalt writers<sup>7,8</sup>. Our strategy was to generate several bistable displays simultaneously on the screen (Fig. 3). Twelve naive subjects viewing these displays reported that all the oscillating dot-pairs seemed to 'lock-in'—that is, the displays always had the same motion axis. This was true even when adjacent displays were separated by several degrees. If one of the displays changed its motion axis then all the displays invariably changed with it simultaneously. This effect suggests the presence of a global field for resolving ambiguity in bistable displays for if the different displays were being processed independently there is no *a priori* reason why their oscillations should become synchronized. The presence of such a field is interesting for two reasons. First, if several Necker cubes or other similar reversible figures are simultaneously present in the visual field then they tend to undergo reversals more or less independently of each other<sup>9</sup>. This suggests that a field probably exists only for certain classes of visual displays such as the motion displays used in our study or the clusters of triangles described by Attneave<sup>1</sup>. Second, the effect seems to be inconsistent with the 'independence-assumption' of Ullman<sup>10</sup> according to which apparent motion should be based on strictly local computations and there ought to be no global field-like effects.

To rule out tracking eye movements as a possible explanation we created two clusters of figures (such as Fig. 3) side-by-side so that one of them was rotated by an angle of exactly 45° in relation to the other in the frontoparallel plane. In this display the effect was still present separately in each of these clusters. Since one cannot track simultaneously in two directions, this result suggests that for each cluster of figures a separate field



**Fig. 5** *a*, The effect of a static occluder on the direction of perceived apparent motion. The bottom dot moves horizontally when the occluder is present. *b*, Same experiment as *a* using lines instead of spots. If the occluder is present the line 'slides' behind it but in the absence of the occluder the line appears to shrink and expand or to recede and advance.

must exist that synchronizes the elements within it. It should be noted, however, that there are at least two different ways in which an apparently field-like effect of this kind could arise. Either it could be based on cooperative interactions between adjacent quasi-independent modules or it could be produced by feedback signals from higher centres introducing the same bias in all the modules.

We then wondered whether an unambiguously moving array of dots in the surround could influence the perception of a single ambiguous figure (similar to that in Fig. 2) in the middle. To explore this we generated Fig. 3*a* again on the CRT screen and confirmed that all the oscillating pairs had become synchronized, as in our previous experiment. We then used long strips of white tape to mask off the lower half of each horizontal row of figures (Fig. 4*a*). A small window was then cut in the middle so that one of the ambiguous figures alone was allowed to become fully visible. By this simple procedure we were able to create unambiguous horizontal motion throughout the surround. Not surprisingly we found that this imposed a slight tendency to see horizontal motion in the central ambiguous figure, but the effect was very small. In fact by using voluntary effort one could easily uncouple the central ambiguous figure from the surround and decide to see vertical motion. Hence the coupling between an unambiguous and an ambiguous figure is probably much weaker than the coupling between adjacent ambiguous figures—although of course we had no simple way of measuring the strength of this coupling. This observation suggests that the synchronization of ambiguous motion displays does not arise due to cooperativity but due to some other more high-level mechanism.

While watching this display we made a curious observation. As pointed out above we could easily uncouple the centre from the surround so that vertical motion was seen. But to our astonishment we found that on some occasions when this happened the dots in the surround also moved vertically to imaginary locations behind the strips of masking tape. Since there is nothing in the retinal stimulus that would lead one to expect this, we have dubbed this illusion 'occult apparent motion' (Fig. 4*b*). Six naive subjects were confronted with this display and they all confirmed that they could see this effect. Interestingly, the effect took time to evolve as when viewing Julesz's random-dot stereograms, and two of the subjects had to be told what to look for, that is, they did not report the effect spontaneously. We then simply masked off the central ambiguous figure and found that the illusion disappeared and was replaced by the appearance of unambiguously horizontal



motion in the surround. The illusion could be seen even if the surrounding figures were several degrees (say 3°) away from the central one. It was as though the particular hypothesis the brain had selected to resolve ambiguity in the centre was being automatically applied throughout the visual field. And in the absence of sufficient evidence the brain resorts to simply postulating the required dots behind the occluders.

An informal observation along these lines was made recently by Shepard<sup>11</sup>. Shepard's stimulus was a vertical bar rapidly alternating with a horizontal bar and one could see motion in either one of two different quadrants. Sometimes voluntary effort was ineffective in switching perceived motion from one quadrant to the other and so he had to occlude suitable portions of the display in order to encourage motion to be seen in the other (non-occluded) quadrant. He reports that on doing this he sometimes continued to see motion behind the occluder for a while but does not state how long this tendency persisted. Our effect (occult apparent motion) may, in a sense be regarded as the spatial equivalent of this persistence effect noted by Shepard.

We find that occult apparent motion is visible only if the SOA is sufficiently long. This may reflect the minimum time required by the brain to invoke the occlusion hypothesis. If the SOA is made smaller than 645 ms (mean for four subjects = 645 ms; s.d. = 84), the effect vanishes and the dots in the surround oscillate horizontally even when the dots in the central ambiguous figure are making vertical excursions. Oddly enough the presence of the masking tape, though helpful, is not essential. If the room lights are switched off, so that the tape is no longer visible, one can still see occult apparent motion. This suggests that under appropriate circumstances one can see apparent motion towards an invisible light spot hidden by an invisible occluder! Subjects reported that a faint subjective outline of the tape was visible corresponding to where it should have been physically present.

One does not have to use ambiguous displays to produce occult motion. The effect can also be seen, though less vividly, in displays such as Fig. 5a in which two light spots in the first frame (A) are alternated with a single light spot in the second frame (B). If the cardboard occluder is not shown then observers always see both spots moving towards the single one and fusing with it as expected. But if the occluder is added, the bottom spot appears to move horizontally to hide behind the occluder although there is no stimulus corresponding to it in frame B. The effect is even more striking if a long thin line is used instead of two separate spots (Fig. 5b). We have shown these displays to six naive subjects and all of them confirmed that they could see the illusion. As in the case of Fig. 3, the percept of the spot sliding behind the occluder was more striking at long SOAs (>500 ms).

The effects reported here, synchronized motion (Fig. 3) and occult apparent motion (Figs 4,5) suggest that spatial induction effects can be very compelling and may play an important role in perception. Whenever the brain applies a rule to resolve ambiguities in a given local region, there is a strong tendency to apply the same rule throughout the visual field and the system will go to tremendous lengths to achieve this.

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1. Attneave, F. *Scient. Am.* **225**, 62-71 (1971).
2. Burt, P. & Sperling, G. *Psychol. Rev.* **88**, 171-195 (1981).
3. Pantle, A. J. & Picciano, L. *Science* **193**, 500-502 (1976).
4. Rubin, E. *Visuell Wahrgenommene Figuren* (Glydendalske, Copenhagen, 1921).
5. Rock, I. *An Introduction to Perception* (Macmillan, New York, 1975).
6. Ramachandran, V. S. & Anstis, S. M. *Vision Res.* **23**, 83-86 (1983).
7. Kohler, W. *Gestalt Psychology* (Liveright, New York, 1929).
8. Koffka, K. *Principles of Gestalt Psychology* (Harcourt, New York, 1935).
9. Long, G. M. & Toppino, T. C. *Perception* **10**, 331-334 (1981).
10. Ullman, S. *The Interpretation of Visual Motion* (MIT Press, Cambridge, 1979).
11. Shepard, R. *Mental Images and Their Transformation* (MIT Press, Cambridge, 1982).

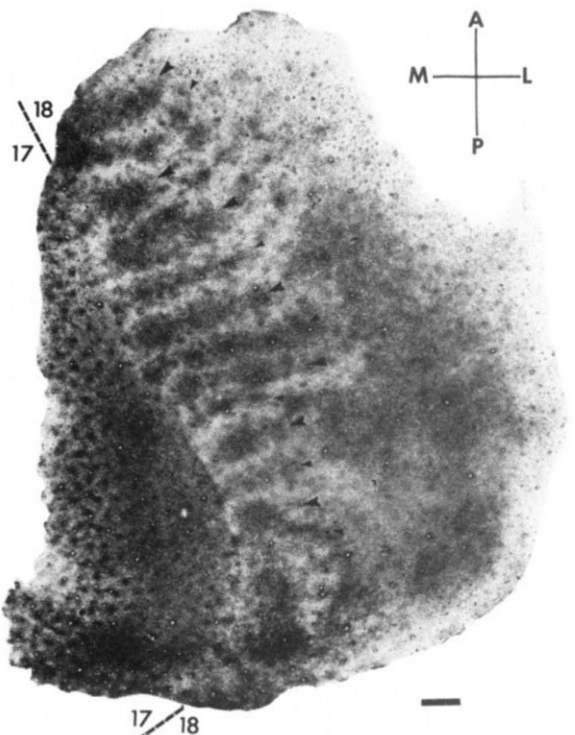
## Specificity of cortico-cortical connections in monkey visual system

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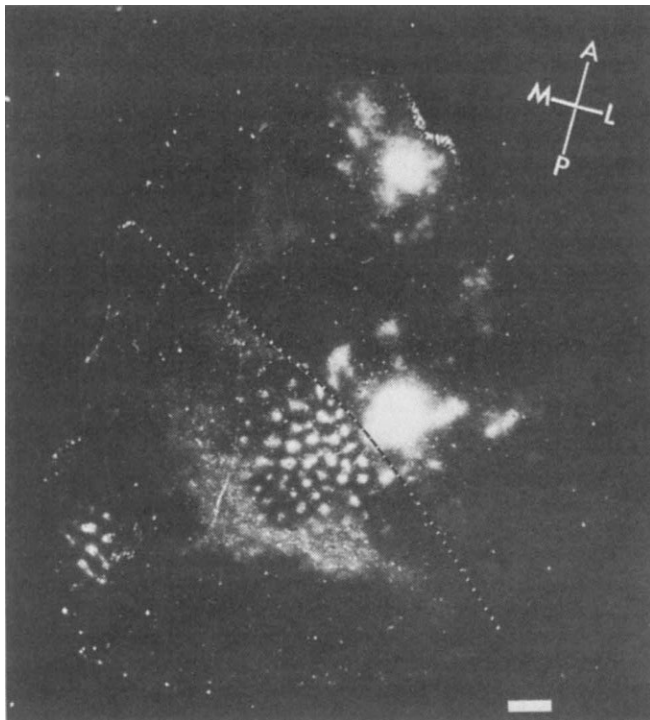
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When the primate primary visual cortex, area 17, is stained for the mitochondrial enzyme cytochrome oxidase<sup>1</sup>, it shows a striking polka-dot pattern (cytochrome oxidase blobs). Area 18, the second visual area, shows a cytochrome-oxidase pattern of coarse alternating thick and thin stripes running perpendicular to the 17-18 border and separated by lighter (interstripe) regions. Here we show that the thin cytochrome oxidase stripes, and possibly also the thick stripes, in area 18 receive projections specifically from the blobs in area 17, and that the interstripe regions of 18 receive projections from the interblob matrix of area 17. This indicates a specificity of cortico-cortical connections far exceeding the demands of topographical mapping. Together with our physiological results, it suggests that within the pathway from area 17 to area 18 different kinds of information may be handled separately and in parallel.

Many major cortical and subcortical areas of the brain are known to be subdivided into columns, stripes or lattices. Areas as diverse as visual, auditory and somatosensory cortices, caudate nucleus and superior colliculus exhibit such subdivisions by local heterogeneities in physiological responses, anatomical connections and staining characteristics<sup>2-6</sup>. M. Wong-Riley (personal communication), using a stain for cytochrome oxidase in area 17 of squirrel monkey, discovered a regular series of densely labelled patches in layers 2 and 3. It was subsequently shown that in sections cut parallel to the surface these patches form a quasiregular array of round or oval blob-like regions 0.2 mm in diameter and spaced 0.5 mm apart<sup>10,11</sup>. In macaque



**Fig. 1** Tangential section through a flattened squirrel monkey occipital lobe. Area 17 is on the left, and 18 on the right. The pattern in 18, although somewhat irregular, is roughly one of alternating thick and thin stripes, as indicated by large and small arrowheads. Scale bar, 1 mm.



**Fig. 2** Tangential section through layers 2-3 of squirrel monkey occipital lobe reacted with tetramethylbenzidine and showing two horseradish peroxidase injection sites in area 18. These are darkfield photographs, taken through crossed polarizers, and the label appears bright. The 17-18 border is indicated by the dotted line. The upper injection site was in an interstripe region in 18, and the lower was in a thin stripe in 18. The cores of the injection sites, not visible in this darkfield photograph, were less than 0.5 mm diameter. The left set of clumps of label in area 17 projected to the upper (interstripe) injection in area 18, and the right set of clumps in 17 projected to the lower (thin stripe) injection in 18. (The confluent grey area below and to the left of the right set of clumps is an artefact.) Scale bar, 1 mm. High-power views are shown in Fig. 3.

monkeys the blobs lie in rows centred on ocular dominance columns<sup>12,13</sup>. Cytochrome oxidase blobs are absent from layer 4C, which stains densely and evenly, but can be seen faintly in layers 4B, 5 and 6; they stain for a variety of other enzymes<sup>13,14</sup>; they are found in all primates so far examined and in no non-primates<sup>12</sup>; they can be seen in fetal brain<sup>14</sup>; they receive direct projections from the lateral geniculate body<sup>15</sup>, probably specifically from intercalated layers<sup>16</sup>; and they are labelled with 2-deoxyglucose after any of a variety of visual stimuli, including, in squirrel monkeys, diffuse light<sup>12,17</sup>. Physiologically, the cells within blobs show no orientation selectivity; many cells show opponent colour responses, and of these the commonest are double-opponent cells, a type that has never been seen in the lateral geniculate body. Cells between blobs, in contrast, are orientation selective, and only a minority are colour coded<sup>18,19</sup>.

Area 18 (visual area II) borders area 17, receives from it a systematic topographical projection mirror symmetric across the 17-18 border, and sends a reciprocal projection back to 17<sup>20-23</sup>. When area 18 is stained for cytochrome oxidase it shows a coarse pattern of parallel stripes running perpendicular to the 17-18 border. These stripes are coincident with the stripes of label produced by 2-deoxyglucose after stimulation with diffuse light<sup>15,24</sup>, and also coincident with stripes formed by the projection from the pulvinar to area 18<sup>15,25,26</sup>. The stripes have a tendency to be alternately wide and narrow, more marked in the deeper layers than in the superficial, and more obvious in some individual animals than in others; the narrow stripes are 0.5-0.7 mm across and come right up to the 17-18

border, where they are most dense, whereas the wide ones are 0.8-1.2 mm across and usually stop 0.5 mm from the 17-18 border (Fig. 1)<sup>15,27</sup>. The total periodicity (for example, thin stripe to thin stripe) is 2.2-2.7 mm. Both types of stripes, especially the thin, tend to be subdivided by paler transverse stripes with a periodicity of 0.75 mm.

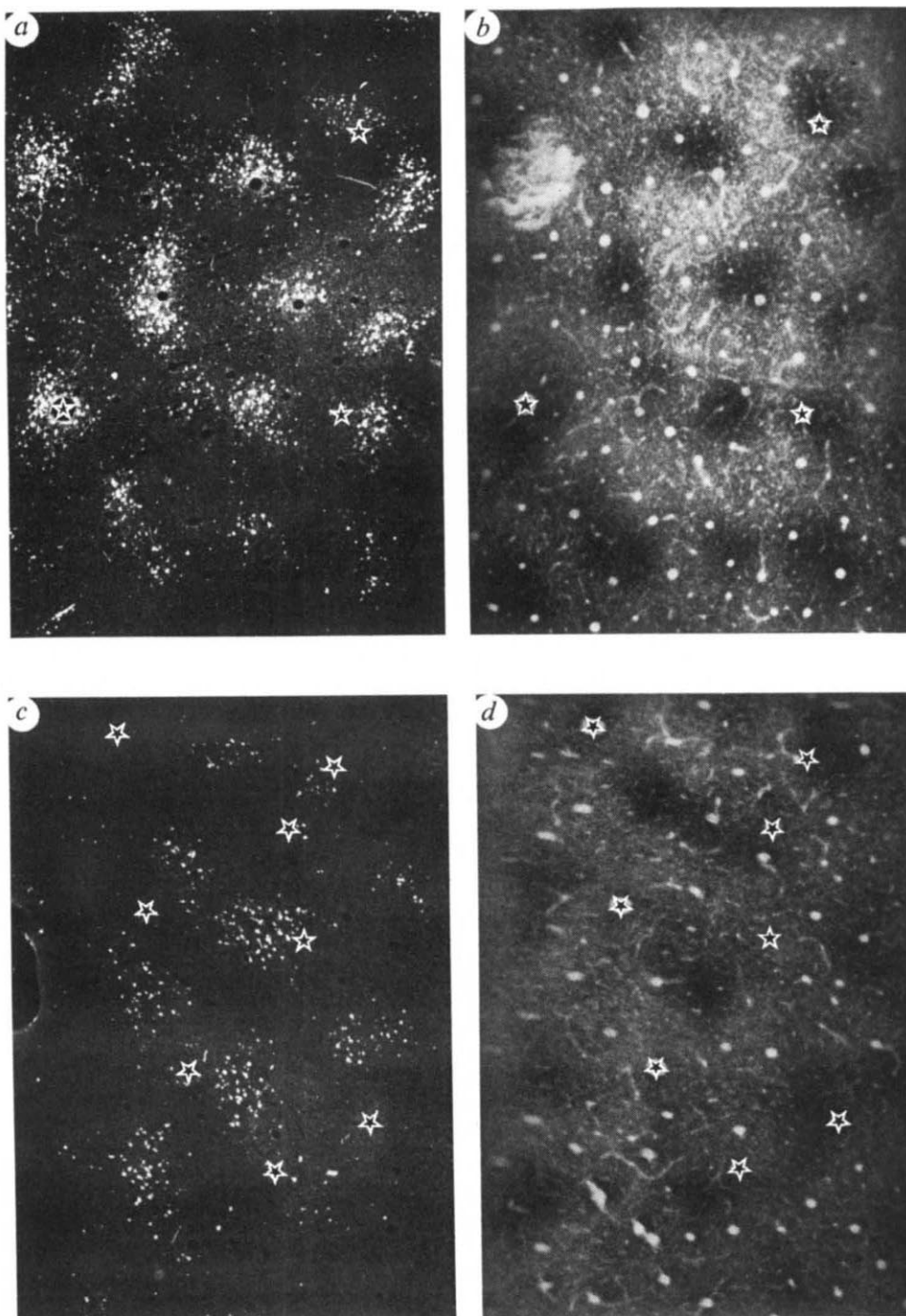
That cells in area 17 projecting to a small region in area 18 can be distributed in small patches has been shown by making small horseradish peroxidase injections into area 18<sup>28,29,34</sup>, but this heterogeneous origin has not been correlated with cytochrome oxidase staining. Three similarities between blobs in area 17 and stripes in area 18 suggested to us that blobs in 17 might selectively project to stripes in 18: first, by definition, both stain for cytochrome oxidase; second, blobs in 17 and stripes in 18 are probably metabolically more active than intervening regions, as indicated by the 2-deoxyglucose results and indeed suggested by the elevated cytochrome oxidase levels; and, finally, both probably receive direct thalamic inputs, from the geniculate to the blobs in area 17 and from the pulvinar to the stripes in 18.

To test the idea that 17 to 18 connections are blob to stripe and interblob to interstripe, we made small iontophoretic injections of wheat-germ agglutinin conjugated to horseradish peroxidase into area 18 of one macaque (5 injections) and seven squirrel monkeys (35 injections). The injections were made blindly, and the locations, whether in a stripe or non-stripe, were determined afterwards histologically. The lectin-horseradish-peroxidase conjugate is transported in both retrograde and anterograde directions<sup>30</sup>. Current (2  $\mu$ A for 5 min, electrode positive) was passed through pipettes with 10-20- $\mu$ m tips filled with 5% lectin-horseradish peroxidase (Sigma) in Tris buffer (0.2 M pH 8.6). Survival time was 48 h. Brains were perfused with mixed aldehydes<sup>31</sup>, flattened and frozen, and alternate tangential sections were reacted for cytochrome oxidase<sup>1</sup> or for horseradish peroxidase<sup>30</sup>. We could align neighbouring sections by overlapping the blood vessels, which run in a precisely radial fashion. Four of the macaque injections and 16 of the squirrel monkey injections were successful in that the central core was localized to either a stripe or an interstripe and produced retrograde filling of cells in area 17. Although we made two to three injections in each hemisphere, the injections were sufficiently widely spaced for the correspondence across the 17-18 border always to be clear from the known topography. The injections were all within 6 mm of the 17-18 border. In seven injections the central core of the injection was confined to a thin stripe, and one injection was in a thick stripe. Each thin stripe injection resulted in a discrete set of labelled patches in area 17; each patch coincided with a cytochrome oxidase blob and consisted of a cluster of densely labelled cells. The one thick stripe injection that produced label in area 17 gave no clear pattern in 17, although there was a tendency for the blob cells to be selectively, but very faintly, labelled. (One other thick stripe injection produced no label in 17, a result that we cannot interpret.) In contrast, all 12 injections whose cores were confined to interstripes produced in area 17 selective labelling of cells outside the blobs. We have assumed that the central core of the injection site, and not the surrounding halo, is the primary source of the transported label, largely because of the cleanliness of our results. Injections in which the core straddled a stripe and a non-stripe resulted in confluent label in area 17.

A low-power tangential section through the flattened cortex of a squirrel monkey is shown in darkfield illumination in Fig. 2. The upper injection site was in an interstripe region in area 18, the lower injection in a thin stripe in 18. Both injections produced a dozen or more clumps of labelled cells in the topographically corresponding region of 17, and also produced puffs of label around the injection site in 18. Figure 3a, c shows the clumps of retrogradely filled cells in area 17 at higher power and Fig. 3b, d the adjacent cytochrome-oxidase stained sections. Figure 3a, b shows the region of area 17 that projected to the thin-stripe injection. Almost all the labelled cells lie in



**Fig. 3** Tetramethylbenzidine reacted section (*a, c*) and adjacent cytochrome oxidase stained section (*b, d*). *a* and *b* show the region in area 17 projecting to the thin stripe injection site in area 18, and *c* and *d* show the region in area 17 projecting to the interstripe injection site. In each pair of photographs the stars mark corresponding blood vessels. In *a* and *b*, labelled cell clusters and cytochrome oxidase blobs coincide, whereas in *c* and *d* the clusters fall in the spaces between blobs. The reader is encouraged to trace on a piece of clear plastic the blood vessel pattern and the labelled patches and then compare the tracing with the cytochrome oxidase pattern. (Because in sections stained for cytochrome oxidase we saw no preferential staining of the injection sites, we assume that horseradish peroxidase does not react in the cytochrome oxidase stain.) Scale bar, 0.5 mm.



blobs. Figure 3c, d shows the part of area 17 projecting to the interstripe injection site; here, in contrast, the labelled cells lie in interblob regions.

After injections into interstripe regions of area 18, there was variation in the distribution of interblob cells labelled in area 17: in some experiments, for instance that of Fig. 3, there were isolated non-blob clusters, but in others the labelled regions formed lines or zig-zags or even a lattice that nearly surrounded but always avoided the blobs.

Following both types of injection, into thin stripe and interstripe regions, there was diffuse fine labelling interspersed between the cells but confined to the regions in which cells were labelled. This probably represents orthograde horseradish peroxidase transport from 18 to 17, and suggests that there are precise sets of reciprocal connections to and from blobs and to and from non-blob regions. It is possible that this fine diffuse label represents axon collaterals of the cells in area 17 labelled by retrograde transport from area 18, but given the known

projection of 18 to 17, it is unlikely that such collaterals would be labelled and not the projections themselves.  $^3\text{H}$ -proline injections into area 18 would probably settle the question as proline is transported only in an anterograde direction.

When the injection site was in a thin stripe, the patches of label in area 18 surrounding the injection also tended to be in stripes (both thick and thin), with the intervening interstripe regions unlabelled. In contrast, when the injection site was in an interstripe region, the patches in area 18 were predominantly in interstripe territory with the intervening stripes blank. Again, label was seen in cell bodies and in the regions between cells, suggesting that reciprocal connections exist between stripe regions and between interstripe regions in area 18. We saw projections between thick stripes, between thin, and between thick and thin. None of these segregations, however, were as complete as those of the 17 to 18 connections.

To summarize, blob cells in layers 2 and 3 of area 17 project to thin stripes in area 18, and probably receive reciprocal inputs

from them. We are not certain whether this is also true of thick stripes. Interstripe regions in area 17 clearly project to interstripe regions in area 18, and it is similarly likely that the reciprocal 18-to-17 projections are segregated.

The present results show by direct anatomical methods that the already well-known orderly interconnections between two cortical areas are more specific than would be required by topographical correspondence alone. Taken together, the likelihood that area 17 forms the main visual input to 18 and the presence in both of orientation-specific cells grouped in columns<sup>32</sup> had already suggested that highly specific connections are likely to link columns of similar orientation selectivity in areas 17 and 18<sup>33</sup>. The interblob to interstripe connections may

indeed be further segregated according to orientation preference. Here, however, we are apparently dealing not simply with one system in which the different values of a variable such as orientation are linked like-to-like between cortical areas, but with two distinct systems, one concerned with orientation and probably other variables, the other oblivious to orientation but concerned, at least to some extent, with colour.

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1. Wong-Riley, M. *Brain Res.* **171**, 11–28 (1979).
2. Mountcastle, V. B. *J. Neurophysiol.* **20**, 408–434 (1957).
3. Hubel, D. H. & Wiesel, T. N. *J. Physiol., Lond.* **160**, 106–154 (1962).
4. Abeles, M. & Goldstein, M. H. Jr *J. Neurophysiol.* **33**, 172–187 (1970).
5. Woolsey, T. A. & Van der Loos, H. *Brain Res.* **17**, 205–242 (1970).
6. Jones, E. G., Burton, H. & Porter, R. *Science* **190**, 572–574 (1975).
7. Shanks, M. F., Rockel, A. J. & Powell, T. P. S. *Brain Res.* **98**, 166–171 (1975).
8. Goldman, P. S. & Nauta, W. J. H. *Brain Res.* **122**, 393–413 (1977).
9. Graybiel, A. M., Ragsdale, C. W. Jr & Moon Edley, S. *Expl Brain Res.* **34**, 189–195 (1979).
10. Horton, J. C. & Hubel, D. H. *Soc. Neurosci.* **6**, 315 (1980).
11. Humphrey, A. L. & Hendrickson, A. E. *Soc. Neurosci.* **6**, 315 (1980).
12. Horton, J. C. & Hubel, D. H. *Nature* **292**, 762–764 (1981).
13. Hendrickson, A. E., Hunt, S. P. & Wu, J.-Y. *Nature* **292**, 605–607 (1981).
14. Horton, J. C. thesis, Harvard Univ. (1983).
15. Livingstone, M. S. & Hubel, D. H. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6098–6101 (1982).
16. Fitzpatrick, D., Itoh, K. & Diamond, I. T. *J. Neurosci.* **3**, 673–702 (1983).
17. Humphrey, A. L. & Hendrickson, A. E. *J. Neurosci.* **3**, 345–358 (1983).
18. Hubel, D. H. & Livingstone, M. S. *Soc. Neurosci.* **7**, 357 (1981).
19. Hubel, D. H. & Livingstone, M. S. *Soc. Neurosci.* **8**, 706 (1982).
20. Talbot, S. A. & Marshall, W. H. *Am. J. Ophthalmol.* **24**, 1255–1263 (1941).
21. Cowey, A. J. *J. Neurophysiol.* **27**, 366–393 (1964).
22. Hubel, D. H. & Wiesel, T. N. *J. Neurophysiol.* **28**, 229–289 (1965).
23. Tiggles, J., Spatz, W. B. & Tiggles, M. J. *comp. Neurol.* **148**, 481–490 (1973).
24. Tootell, R. B. H. & Silverman, M. S. *Soc. Neurosci.* **11**, 356 (1981).
25. Rezak, M. & Benevento, L. A. *Soc. Neurosci.* **6**, 1088 (1976).
26. Curcio, C. A. & Harting, J. K. *Brain Res.* **143**, 155–161 (1978).
27. Tootell, R. B. H., Silverman, M. S., DeValois, R. L. & Jacobs, G. H. *Science* **220**, 737–739 (1983).
28. Maunsell, J. H. R., Newsome, W. T. & Van Essen, D. C. *Soc. Neurosci.* **6**, 580 (1980).
29. Tiggles, J. et al. *J. comp. Neurol.* **202**, 539–560 (1981).
30. Mesulam, M.-M. in *Tracing Neural Connections with Horseradish Peroxidase* (ed. Mesulam, M.-M.) 1–151 (Wiley, New York, 1982).
31. Graham, R. C. Jr & Karnovsky, M. J. *J. Histochem. Cytochem.* **14**, 291–302 (1966).
32. Hubel, D. H. & Wiesel, T. N. *J. Physiol., Lond.* **195**, 215–243 (1968).
33. Hubel, D. H. & Wiesel, T. N. *J. comp. Neurol.* **158**, 267–294 (1974).
34. Wong-Riley, M., *Brain Res.* **147**, 159–164 (1978).

## Processing of polarized light by squid photoreceptors

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**Behavioural tests<sup>1–4</sup> have demonstrated that cephalopods can discriminate light polarized in different planes, and the receptors have been localized by electrophysiological studies of the eye<sup>5–10</sup>. Discrimination of the plane of polarization is a consequence of both the structure of the microvilli in the outer segments of the photoreceptors<sup>11</sup> and the orientation of the photosensitive chromophore on these membranes<sup>2,12,13</sup>. However, between the depolarizing receptor response resulting from photoreception and the behaviour of the animal, nothing is known about neuronal processing of polarized light by cephalopods. Here we show that some squid photoreceptors discriminate the plane of polarization within the spike train, and that any particular plane is seen as a variable intensity. Given the well known orthogonal orientation of microvilli in outer segments of adjacent photoreceptors and the physiological preference for one of two mutually perpendicular planes of polarization by single photoreceptors, we conclude that cephalopod vision is based on two complementary views of the world, each determined by the transformation of polarization-sensitive receptors into complementary intensity scales. A visual system based on this transformation would lead to enhanced contrast underwater and visualization of object details obscured by confounding highlights.**

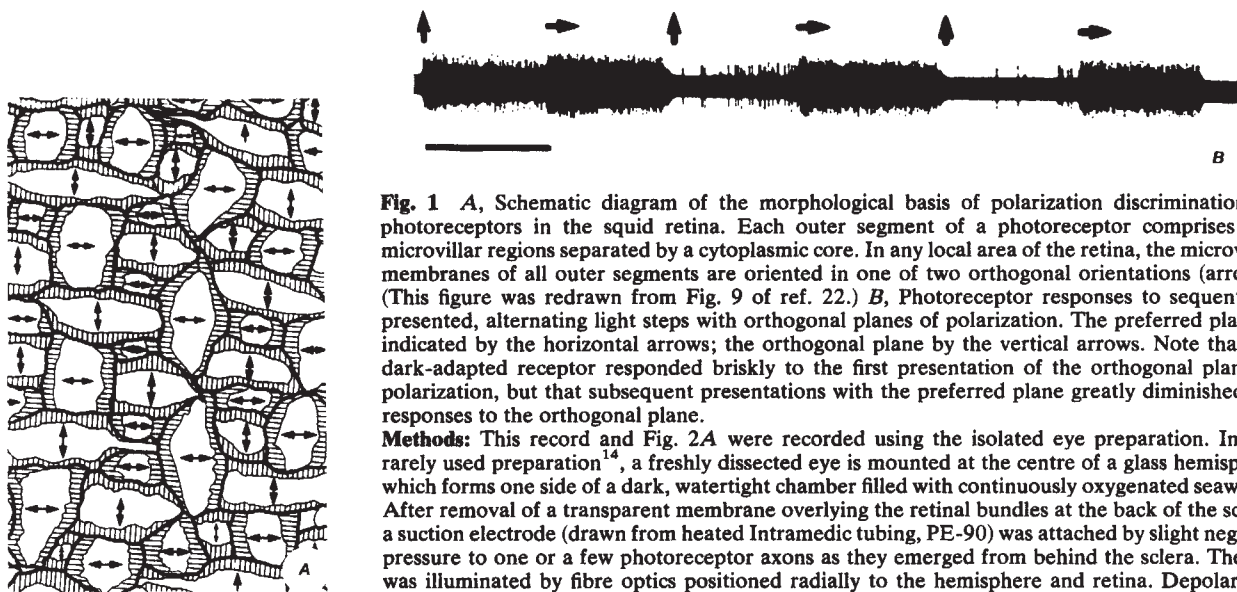
Individual and multi-unit spikes were recorded from photoreceptor axons in viable, isolated eye preparations<sup>14</sup> of the

squid, *Loligo pealei*. A spike response of a squid photoreceptor to a step of increased illumination is a train of impulses in which the onset time and spike rate depend on the intensity of the incident illumination. The train reflects the generator potential, that is, the change in transmembrane potential resulting from photon capture by the chromophore, in that it consists of an initially fast spike rate (for <1 s) followed by a slower steady-state rate. The presence of the initial fast rate depends on exceeding a threshold, a phenomenon also seen for the early phase of the depolarizing generator potential<sup>15</sup>. The steady-state response encodes the plane of polarization of the stimulus as shown below. Thus, the two-phased behaviour of the generator potential<sup>15</sup> is reflected in the two-phased behaviour of the spike train.

We found that the initial presentation of a polarized light stimulus of any plane to a dark-adapted photoreceptor elicits a brisk response. Using spike rate (spikes per s) during the steady-state phase as a measure, one specific polarization plane was typically most effective (defined here as the 0° or 'preferred' plane). In the experiments reported, we observed only two preferred planes of polarization in a local patch of retina and they were orthogonal to each other. These preferences are probably defined by the orthogonal microvilli seen in adjacent photoreceptor outer segments<sup>16–20</sup> (Fig. 1A). Figure 1B illustrates the responses to a sequential presentation of light linearly polarized in the orthogonal and preferred planes. A photoreceptor's response to the preferred plane was more resistant to the effects of light adaptation than the response to a stimulus with another plane of polarization. The steady-state response to the preferred plane showed a small decrement due to the adaptive consequences of repetitive stimulation while the steady-state response to a stimulus having the orthogonal plane of polarization adapted to nearly no response. This response is just as would be predicted if the preferred orientation were seen as a brighter source.

Figure 2 illustrates how these photoreceptors discriminate the plane of polarization. Figure 2A shows responses to light polarized in the preferred plane (0°), polarized at 45° and at 90° to the preferred plane by the same photoreceptor, dark-adapted (left) and light-adapted (right). The total number of spikes between the second and eighth seconds of a 10-s light stimulus were compared with the number of spikes during the same interval elicited by light polarized in the preferred plane





**Fig. 1** *A*, Schematic diagram of the morphological basis of polarization discrimination by photoreceptors in the squid retina. Each outer segment of a photoreceptor comprises two microvillar regions separated by a cytoplasmic core. In any local area of the retina, the microvillar membranes of all outer segments are oriented in one of two orthogonal orientations (arrows). (This figure was redrawn from Fig. 9 of ref. 22.) *B*, Photoreceptor responses to sequentially presented, alternating light steps with orthogonal planes of polarization. The preferred plane is indicated by the horizontal arrows; the orthogonal plane by the vertical arrows. Note that the dark-adapted receptor responded briskly to the first presentation of the orthogonal plane of polarization, but that subsequent presentations with the preferred plane greatly diminished the responses to the orthogonal plane.

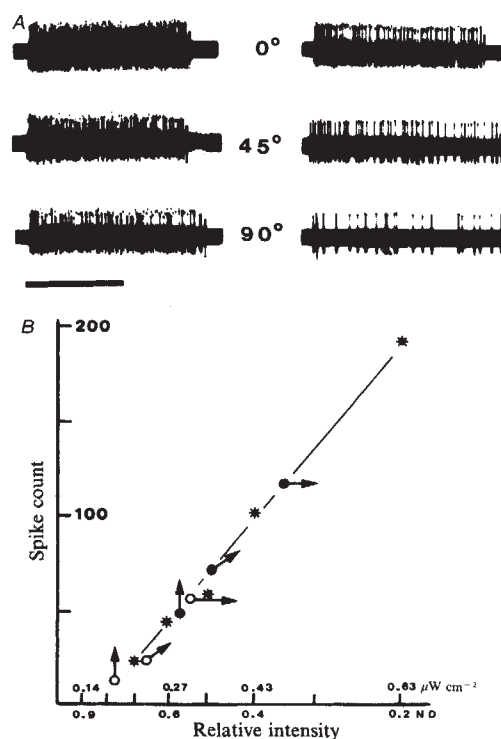
**Methods:** This record and Fig. 2A were recorded using the isolated eye preparation. In this rarely used preparation<sup>14</sup>, a freshly dissected eye is mounted at the centre of a glass hemisphere which forms one side of a dark, watertight chamber filled with continuously oxygenated seawater. After removal of a transparent membrane overlying the retinal bundles at the back of the sclera, a suction electrode (drawn from heated Intramedic tubing, PE-90) was attached by slight negative pressure to one or a few photoreceptor axons as they emerged from behind the sclera. The eye was illuminated by fibre optics positioned radially to the hemisphere and retina. Depolarizing and linearly polarizing filters and/or neutral density filters were placed between the fibre optics and the hemisphere to test a photoreceptor's responses to unpolarized and linearly polarized light stimuli of differing planes. (Angular references to the planes of polarization are relative to each other within a stimulus set.) Scale bar, 10 s.

( $R/R_{\text{pref}}$ ) to determine the relative efficiency of light polarized in a variety of planes. When dark-adapted, the effectiveness in eliciting spikes by linearly polarized light steps whose plane of polarization were orthogonal and  $45^\circ$  to the preferred plane, was 0.45 and 0.62, respectively. These ratios decreased to 0.19 and 0.39, respectively, for the light-adapted photoreceptor. In addition, the photoreceptor when light-adapted decreased its response compared with the dark-adapted condition; the rates decreased by 49, 79 and 67% to light steps having the preferred plane, the orthogonal plane, and a diagonal plane ( $45^\circ$ ) respectively.

The spike response to unpolarized light varies directly with the log of the intensity over about one decade (Fig. 2B). Each response to polarized light of different planes of polarization can thus be directly matched to an 'equivalent intensity'. The spiking properties of squid photoreceptors to non-optimally polarized light stimuli may then be described as the response to the most favourable plane of polarization but with an intensity that represents the diminished efficiency of the analysers within the photoreceptor (the microvillar membrane) to non-optimal planes of polarization. Figure 2A illustrates that the microvilli do not act as perfect analysers as the response to a stimulus orthogonal to the preferred is not zero, as would be expected for light transmission through orthogonally-crossed Polaroids. Moreover, as reported previously<sup>14</sup>, we observed a complete spike cessation when the intensity increased dramatically over the operating range of the photoreceptor.

These experimental results imply that the two classes of photoreceptors seen in electron micrographs<sup>2,16-20</sup> reflect the preferred physiological polarization sensitivities of the cephalopod retina. The neural behaviour of these cells suggests that cephalopod photoreceptors process information about the plane of polarization in a fashion analogous to the processing of chromatic information in colour vision by the different vertebrate cones, that is, by transforming the stimulus to which the receptor responds into an intensity scale<sup>21</sup>. Vision in the cephalopod eye, however, is based on two measures, not three as in the vertebrate eye.

The relatively simple anatomical picture of the cephalopod retina and its physiological responses is complicated by: (1) the close proximity of adjacent outer segments in the so-called 'rhabdom' of the cephalopod eye<sup>2,16-20</sup>; (2) the fine processes extending between inner segments of nearby photoreceptors<sup>22</sup>; and (3) centrifugal cells located within the optic lobe forming axosomatic synapses onto both classes of photoreceptors<sup>23</sup>. The



**Fig. 2** *A*, Dark- (left) and light- (right) adapted responses to light stimuli whose plane of polarization was set to the preferred ( $0^\circ$ ), orthogonal ( $90^\circ$ ), or mid-way between the two planes ( $45^\circ$ ). Scale bar, 5 s. *B*, This graph illustrates the exponential nature of the spike response as a function of light intensity. The energy of the unfiltered stimulus was measured with a Tektronix J16 digital photometer at  $1.09 \mu\text{W cm}^{-2}$ ; measured after attenuation by a Polaroid filter in the preferred and orthogonal planes of polarization at  $0.63 \mu\text{W cm}^{-2}$ , and after attenuation by a Kodak Wratten 0.2 ND filter at  $0.62 \mu\text{W cm}^{-2}$ . The responses to polarized light identify the equivalent intensity to the different planes in the dark- and light-adapted conditions. The spike response is the count of all spikes between 2.00 and 8.99 s. The unpolarized, but variously attenuated stimuli are starred; the dark-adapted responses are indicated by filled circles; and the light-adapted responses by open circles. Arrows indicate the preferred plane of polarization (horizontal); the plane of polarization at  $45^\circ$  (diagonal); and the plane at  $90^\circ$  (vertical).

physiological effects of these anatomical complexities are unknown; they may be involved in both dynamic and static lateral interactions<sup>5,24,25</sup>. More detailed studies using the isolated eye or isolated eye-optic lobe preparation<sup>14</sup> should elucidate these effects.

Differences in the sharpness of differentiating one particular polarization plane from another by photoreceptors may be provided by two factors: (1) the differences in microvillar area among single photoreceptors (Fig. 1A); and (2) the pigment granules found in the cytoplasmic core of the outer segment and the adjacent granules found in the cytoplasmic core of the outer segment and the adjacent supporting cells<sup>5,26,27</sup>. In dark-adapted retinas, the granules are located adjacent to the basement membrane; they migrate distally during light reception<sup>26,27</sup>. The light-induced change in response characteristics of photoreceptors shown in Fig. 2A in part originates from the migration of these granules. When light-adapted, the migrated pigment granules would absorb a certain percentage of the incident illumination, thus enhancing the probability of detection of polarized light with the preferred plane over polarized light of any other plane.

Most studies that have analysed the visual capabilities of cephalopods<sup>28</sup> have ignored polarization as a major consideration in perceptual processes. Current thoughts on the utility of polarization detection in other invertebrates centre around spatial orientation or polarotaxis<sup>11</sup>. We suspect that changes in postural orientation that follow changes in the dominant plane of polarized light underwater<sup>4</sup> (evidence interpreted as a demonstration of polarotaxis) may aid visual perception by allowing the animal to orient for the most favourable view in which to observe objects against the partially polarized space light<sup>29,30</sup>.

The results we report raise an important question: what is the utility of distributing the detection of polarization at a resolution scale<sup>31</sup> nearly equivalent to that which some vertebrates use for colour vision? Our results argue that adjacent photoreceptors having overlapping receptive fields would see the same object, but that the object might appear to the photoreceptors as differing in intensity depending on the nature of the reflected, polarized light. This raises the interesting and uninvestigated problem of central integration of the information derived by the two sets of orthogonally sensitive photoreceptors.

On the whole, knowing the plane of polarization in the illuminant does not generate such detailed information. We have speculated that this system provides cephalopods with a significant advantage, which we can appreciate by making a simple observation on ourselves. If we wear a pair of polarized lenses in which the right and left lenses have orthogonal preferences of planes of polarization, we notice that some surfaces reflect a strongly polarized highlight. In one eye alone, this highlight obscures the details of the surface, while in the other eye, the obscured details are visible. Since, in binocular vision, edges and detail take precedence over featurelessness, the details are observed when the two views are binocularly fused. For humans, glasses polarized in this fashion have obvious utility. We believe that this 'binocular' interaction occurs in octopus and squid but with one major difference. Whereas in humans, the binocular interaction originates from the orthogonally polarized views seen by both eyes, in cephalopods the physiological and anatomical evidence argues that the 'orthogonally polarized views' are interdigitated within the retina of a single eye.

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7. Tasaki, K. & Karita, K. *Jap. J. Physiol.* **16**, 205–216 (1966).
8. Tomita, T. *Proc. IEEE* **56**, 1015–1023 (1968).
9. Saidel, W. M. *Biol. Bull.* **159**, 490 (1980).
10. Suguwara, L., Katagiri, Y. & Tomita, T. *J. Fac. Sci. Hokkaido Univ. Ser. VI* **17**, 581–586 (1971).
11. Waterman, T. H. in *Handbook of Sensory Physiology* Vol 7/6B (ed. Autrum, H.) 281–469 (Springer, Berlin, 1981).
12. Tauber, U. in *Photoreceptor Optics* (eds Snyder, A. W. & Menzel, R.) 296–315 (Springer, Berlin, 1975).
13. Schlecht, P. & Tauber, U. in *Photoreceptor Optics* (eds Snyder, A. W. & Menzel, R.) 316–335 (Springer, Berlin, 1975).
14. Lange, G. D. & Hartline, P. H. *J. comp. Physiol.* **93**, 19–36 (1974).
15. Pinto, L. H. & Brown, J. E. *J. comp. Physiol.* **122**, 242–250 (1977).
16. Wolken, J. J. *J. biophys. biochem. Cytol.* **4**, 835–838 (1958).
17. Young, J. Z. *Nature* **186**, 836–839 (1960).
18. Zonana, H. V. *Bull. Johns Hopkins Hosp.* **100**, 185–205 (1961).
19. Yamamoto, T., Tasaki, K., Sugawara, Y. & Tonosaki, A. *J. Cell Biol.* **25**, 345–359 (1965).
20. Cohen, A. I. *J. comp. Neurol.* **147**, 351–377 (1973).
21. Bernard, G. D. & Wehner, R. *Vision Res.* **17**, 1019–1028 (1977).
22. Young, J. Z. *Phil. Trans. R. Soc. B* **245**, 1–18 (1962).
23. Lund, R. D. *Expl Neurol.* **15**, 100–112 (1966).
24. Lange, G. D., Hartline, P. H. & Hurley, A. C. in *Neural Principles in Vision* (eds Zettler, F. & Weiler, R.) 389–393 (Springer, Berlin, 1976).
25. Saidel, W. M. *Soc. Neurosci. Abstr.* **5**, 260 (1979).
26. Young, J. Z. *Proc. zool. Soc. Lond.* **140**, 255–272 (1963).
27. Daw, N. W. & Pearlman, A. L. *J. gen. Physiol.* **63**, 22–36 (1974).
28. Wells, M. J. *Octopus: Physiology and Behavior of an Advanced Invertebrate*, 179–216 (Chapman & Hall, London, 1978).
29. Lythgoe, J. N. & Hemmings, C. C. *Nature* **213**, 893–894 (1967).
30. Land, M. F. in *Handbook of Sensory Physiology* Vol 7/6B (ed. Autrum, H.) 520 (Springer, Berlin, 1981).
31. Packard, A. *Monitore zool. ital.* **3**, 19–32 (1969).

## Frequency tuning in a frog vestibular organ

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Several distinct mechanisms have evolved in the auditory periphery to extract frequency information from a sound. In the mammalian cochlea, a travelling wave on the basilar membrane<sup>1</sup> enhanced by a physiologically vulnerable neuromechanical interaction<sup>2,3</sup> performs the primary frequency separation. In lizards, tuning is likely to depend on structures in the papilla other than the basilar membrane<sup>4</sup>, and tuning in the auditory nerve is correlated with the length of the stereocilia<sup>5</sup>. In turtles<sup>6</sup> and possibly some bird species<sup>7</sup>, an electrical resonance in the receptor cells is responsible for frequency selectivity. In addition to those organs obviously specialized to detect acoustic stimuli, afferents of the vestibular system can exhibit tuning to low-frequency airborne sounds<sup>8,9</sup>, despite the absence of mechanical frequency separation by accessory structures. I report here that in the frog sacculus, a vestibular organ apparently constructed for the detection of vibratory accelerations<sup>8–10</sup>, frequency tuning may arise from an electrical resonance intrinsic to the hair cells. The mechanism is similar to that found in turtle<sup>6</sup> and ensures that a stimulus with frequency corresponding to the membrane resonant frequency will produce the largest signal in the cell. This type of tuning may thus be quite widespread. Oscillatory mechanisms have been reported in sensory cells of other modalities in several lower vertebrates<sup>6,11,12</sup>, and may even contribute to their sensitivity, although such mechanisms do imply that the signal-to-noise ratio is degraded near threshold.

Isolated neuroepithelia from the sacculus of the frog *Rana pipiens* were mounted on the stage of a microscope<sup>13</sup> following removal of the otoconial mass and otolithic membrane by mild collagenase treatment<sup>14</sup>. Individual hair cells were impaled under visual control by potassium acetate-filled microelectrodes of impedance 140–200 MΩ. Cells were maintained in Ringer's solution (Na<sup>+</sup>, 113 mM; K<sup>+</sup>, 2 mM; Ca<sup>2+</sup>, 4 mM; Cl<sup>−</sup>, 123 mM; HEPES, 5 mM; pH 7.3). The efferent synapse in the cells was functionally intact<sup>13</sup>, and stimulation of the saccular nerve gen-

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1. Moody, M. F. & Parriss, J. R. *Nature* **186**, 839–840 (1960).

2. Moody, M. F. & Parriss, J. R. *Z. vergl. Physiol.* **44**, 268–291 (1961).

3. Rowell, C. H. F. & Wells, M. J. *J. exp. Biol.* **38**, 827–831 (1961).

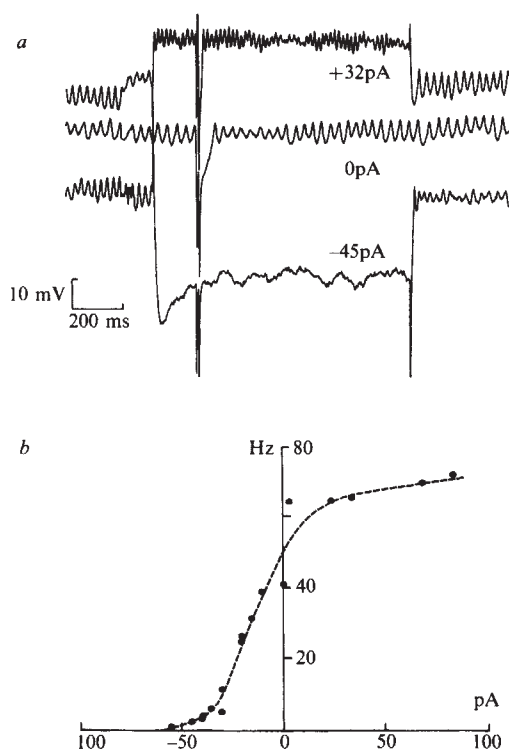
4. Jander, R., Daumer, K. & Waterman, T. H. *Z. vergl. Physiol.* **46**, 383–394 (1963).

5. Lettvin, J. Y. Q. *Prog. Res. Lab. Electron. M.I.T.* **64**, 288–290 (1962).

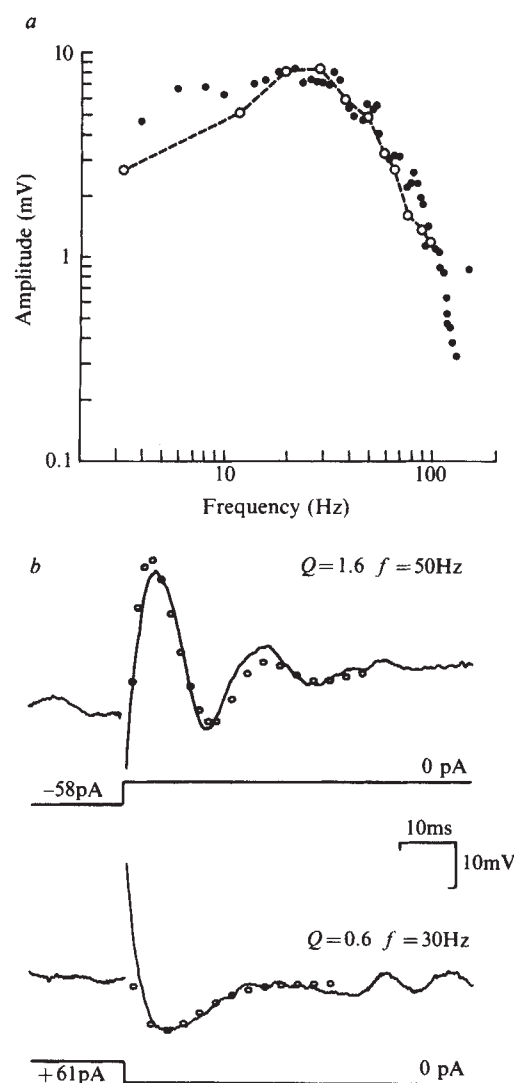
6. Tasaki, K. & Karita, K. *Nature* **209**, 934–935 (1966).

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**Fig. 1** Spontaneous membrane potential oscillations in saccular hair cells. The middle trace in *a* shows large spontaneous membrane potential oscillations at the resting potential. Extrinsic current passed through the electrode using an active bridge altered the frequency (top and bottom traces). The cell input resistance was 560 M $\Omega$  at the resting membrane potential of -65 mV. The double artefacts 200 ms after the current onset are shocks delivered to the saccular nerve which elicited an efferent i.p.s.p.<sup>13</sup>. The i.p.s.p. altered in time course with membrane polarization<sup>20</sup>. The spontaneous oscillation frequency increased slightly following the i.p.s.p. The dependence of spontaneous oscillation frequency on injected current is shown in *b*. The nonlinear dependence matches the *I-V* curve for the cell. Temperature 19 °C.



**Fig. 2** Tuning in saccular hair cells. *a*, Peak amplitudes of the receptor potentials (open circles) generated by a 1.1  $\mu$ m sinusoidal displacement of the stereocilia by a glass microprobe driven by a piezoelectric element at different frequencies. The probe motion was checked for constant amplitude when uncoupled from the hair bundle using a laser source illumination and a photodiode. The displacement was constant over 5–100 Hz to within 4%. Although the stereocilial motion was not measured directly, the hair cell receptor potentials followed a triangular stimulus at low frequencies. Additional roll-off of the receptor potentials above 50 Hz may arise from compliance in the coupling of the probe, but would not affect the frequency of the peak receptor potential. At the lowest frequencies (<8 Hz) adaptation of the receptor potential reduced the amplitude of the response equivalent to a high-pass filtering of the response<sup>21</sup>. The closed circles show magnitudes of the Fourier components of the spontaneous membrane potential fluctuations, computed from 10 s of record low-pass Butterworth filtered with a cutoff frequency of 100 Hz and digitized at 500 Hz into 256 point frames. The fast Fourier transform was performed on a small desktop computer. The amplitude spectrum has been scaled to superimpose it on the tuning curve. Variance of the spontaneous noise, 0.39 mV<sup>2</sup>. The contribution from the electrode outside the cell (noise variance, 0.047 mV<sup>2</sup>) has been subtracted from the Fourier components. The time constant of the electrode and recording system was 1.5 ms. *b*, Shows the response of a cell when an extrinsic current step was turned off and shows a highly damped oscillatory component. The open circles show the computed response for the equivalent circuit described in the text, with *Q* and centre frequency chosen as indicated<sup>6</sup>. The peak of the noise spectrum occurred at 52 Hz in this cell. The lower frequency and *Q* obtained for recovery from a depolarizing step (lower trace) probably arises from the rectification of the cell membrane when depolarized from rest<sup>15</sup>.

erated a hyperpolarizing postsynaptic potential in most of the cells described below. Receptor potentials were produced in the cells when the stereocilia were deflected using a glass microprobe inserted between the kinocilium and the stereocilia and driven by a piezoelectric element. In the absence of direct sensory stimulation, a high noise level was observed in many cells. The following observations are based on 16 cells which exhibited a peak-to-peak noise level greater than 2 mV. The noise in some cases took the form of a spontaneous oscillation (Fig. 1*a*). The cell shown in Fig. 1 oscillated spontaneously at 41 Hz, with a peak amplitude of 7 mV but with a low-frequency amplitude modulation at about 2 Hz. The oscillation frequency was voltage-dependent, being reduced to near 0 Hz by hyperpolarizing current and increased by depolarizing current (Fig. 1*b*). The sigmoidal shape of the curve of frequency against current followed closely the shape of the current-voltage (*I-V*) curve for the cell<sup>15</sup>, and may indicate that the frequency of the oscillations is linearly dependent on membrane potential. The noise variance of this cell was 3.54 mV<sup>2</sup>. In other cells, the total noise variance ranged over 0.249–2.93 mV<sup>2</sup>, with electrode contribution below about 0.05 mV<sup>2</sup>.

A similar spontaneous membrane potential fluctuation has been described in the hair cells of the turtle cochlea<sup>6</sup>. In the turtle, the acoustic best frequencies are matched by a peak in the noise spectrum and the frequency at which the cell oscillates when extrinsic current steps are applied. In analogy with an acoustic stimulus, Fig. 2*a* shows an experiment in which the sensory hair bundle was stimulated by a direct displacement at different frequencies. The amplitude of the receptor potential

was greatest near 34 Hz, and decreased with increasing stimulus frequency. The peak of the amplitude spectrum of the spontaneous noise recorded in the cell occurred near 33 Hz and the high frequency slope indicates that the membrane behaves as a simple low-pass filter above this frequency. The frequency at which the receptor potential peaked could be reduced by hyperpolarizing current, but was unaffected by reduction of  $\text{Ca}^{2+}$  in the bath to 1.8 mM.

The agreement between the frequencies at the two peaks supports the notion of an electrical, rather than mechanical<sup>4</sup> resonant mechanism in the cell membrane which enhances the receptor current at an optimal frequency. Further support for an electrical origin of the tuning arises from experiments in which current was injected into the cell (Fig. 2b). At the start of a current pulse of either polarity, the membrane potential oscillated, with a highly damped return to the resting potential. This oscillation could be fitted by a model in which the cell contained an inductive element,  $L$ , in series with the cell resistance,  $R$ , and in parallel with the membrane capacitance,  $C$ . This simple resonant circuit<sup>6</sup> gives a quality factor  $Q = (L/C)^{1/2}/R$  for the tuning curve. The  $Q$  value of the cell resonant circuit was obtained by fitting the noise power spectra of the spontaneous membrane potential fluctuations to one of a family of curves predicted by the electrical circuit. The  $Q$  values obtained ranged between 0.7 and 3 (mean 1.28,  $n = 16$ ), with the centre frequencies of the power ranging over 11–85 Hz (mean 36.4 Hz). Resting potential (–67 mV, range –58 to –76 mV) was poorly correlated with centre frequency except that the cell with the lowest centre frequency (11 Hz) also had a low resting potential (–76 mV). The larger values of  $Q$  were associated with higher centre frequencies. The  $Q$  values obtained are smaller than those described in the turtle cochlea<sup>5</sup>, but are reduced in proportion to the centre frequencies found in the frog.

Lewis *et al.*<sup>8</sup> have reported that bullfrog saccular afferents are tuned to low-frequency airborne sounds. Such stimuli are inevitably coupled through the ground. Best frequencies in the range 60–160 Hz were reported but vibration-driven afferents may be tuned as low as 15 Hz<sup>9</sup>. Although the range of frequencies reported here overlap with the measurements *in vivo*, the absence of higher centre frequencies may be due to species differences or a difference in the population of sampled cells. In the present case, cells were predominantly selected from the peripheral margin of the neuroepithelium. It is also possible that removal of the otoconial mass leads to a change in the measured electrical tuning, a result suggested if the transducer mechanism is bidirectional<sup>16</sup>, because the cell's measured electrical properties would then depend on the mechanical environment and loading of the sensory hair bundle.

The origin of the spontaneous fluctuation is unlikely to be thermal motion of the air bundle<sup>17</sup>. If the mechanism producing the spontaneous oscillation was a motion of the sensory hair bundle, the r.m.s. values of the spontaneous membrane fluctuation, 0.50–1.88 mV, suggest that the motion should be observable. The measured mean sensitivity<sup>14</sup> of the cells in this sample,  $7 \text{ mV } \mu\text{m}^{-1}$  displacement of the stereocilia, implies that the corresponding motion of the stereocilia tips should be  $0.27 \mu\text{m}$  r.m.s. The potential oscillations of the cell in Fig. 1 would have corresponded to a peak motion of the sensory hair bundle of nearly  $1 \mu\text{m}$  in amplitude. Such motion of the sensory hair bundle would blur the regular pattern of stereocilia observed under high magnification. Energetic considerations also suggest that thermal motion is small. The r.m.s. angular motion of the stereocilia bundle is  $(kT/D)^{1/2}$  where  $k$  is Boltzmann's constant,  $T$  the absolute temperature and  $D$  the restoring torque of the hair bundle<sup>18</sup>.  $D$  is  $\sim 3 \times 10^{-9} \text{ dyn-cm}$  measured photographically from motion of a saccular hair bundle  $6 \mu\text{m}$  long<sup>19</sup>. Thus, the r.m.s. angular motion would be  $0.21^\circ$ , corresponding to an r.m.s. signal in the cell of  $\sim 0.15 \text{ mV}$ , less than the noise observed. Coupling of many stereocilia through the otolithic membrane would further reduce the significance of thermal noise in transduction<sup>17</sup>.

Because the cells received an efferent synaptic input<sup>13</sup>, a component of the noise may have arisen from transmitter mechanisms at the basal surface of the cell rather than the transducer at the apical surface. It was found that 1 mM  $\text{Co}^{2+}$  added to the bath was sufficient to abolish the noise, possibly by blocking transmitter release from the efferent synapse. A comparable effect would arise if the channels described by the inductive element  $L$  were sensitive to  $\text{Co}^{2+}$ . In both cases, however, the dependence of the tuning parameters on membrane potential suggest a subtle interaction between efferent and afferent information flow in saccular hair cells.

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1. v. Bekesy, G. *Experiments in Hearing* (McGraw-Hill, New York, 1960).
2. Davis, H. *Hearing Res.* **9**, 79–90 (1983).
3. Sellick, P. M., Patuzzi, R. & Johnstone, B. M. *J. acoust. Soc. Am.* **72**, 131–141 (1982).
4. Weiss, T. F., Peake, W. T., Ling, A. & Holton, H. in *Evoked Electrical Activity in the Auditory Nervous System* (eds Naunton, R. F. & Fernandez, C.) 91–112 (Academic, London, 1978).
5. Turner, R. G., Muraski, A. A. & Nielson, D. W. *Science* **213**, 1519–1521 (1981).
6. Crawford, A. C. & Fettiplace, R. *J. Physiol., Lond.* **312**, 377–412 (1981).
7. Manley, G. A. *Naturwissenschaften* **66**, 582–84 (1979).
8. Lewis, E. R., Leverenz, E. C. & Koyama, H. *J. comp. Physiol.* **145**, 437–445 (1982).
9. Koyama, H., Lewis, E. R., Leverenz, E. L. & Baird, R. A. *Brain Res.* **250**, 168–172 (1982).
10. Moffat, J. M. & Capranica, R. J. *comp. Physiol.* **105**, 1–8 (1976).
11. Clusius, W. T. & Bennett, M. V. L. *J. gen. Physiol.* **69**, 145–182 (1977).
12. Fain, G. L., Gerschenfeld, H. M. & Quandt, F. J. *Physiol., Lond.* **303**, 495–513 (1980).
13. Ashmore, J. F. & Russell, I. J. *J. Physiol., Lond.* **329**, 26P–27P (1982).
14. Hudspeth, A. J. & Corey, D. P. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2407–2411 (1977).
15. Corey, D. P. & Hudspeth, A. J. *Nature* **281**, 675–677 (1979).
16. Weiss, T. F. *Hearing Res.* **7**, 353–360 (1982).
17. Harris, G. G. *J. acoust. Soc. Am.* **44**, 176–186 (1968).
18. Sommerfeld, A. *Thermodynamics and Statistical Mechanics* (Academic, New York, 1956).
19. Ashmore, J. F. & Russell, I. J. *J. submicrosc. Cytol.* **15**, 163–166 (1983).
20. Art, J. J., Crawford, A. C., Fettiplace, R. & Fuchs, P. *Proc. R. Soc. B* **216**, 377–384 (1982).
21. Eatock, R. A., Corey, D. P. & Hudspeth, A. J. *Soc. Neurosci. Abstr.* **5**, 19 (1979).

## Voltage- and ion-dependent conductances in solitary vertebrate hair cells

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An important function of the peripheral auditory system is the resolution of complex sounds into their constituent frequency components. It is well established that each mechanoreceptive hair cell of the cochlea is maximally sensitive to a particular frequency of sound<sup>1,2</sup>, but the mechanisms by which this sharp frequency selectivity is achieved are still controversial<sup>3</sup>. The complex mechanical and hydrodynamic properties of the receptor organs and of the hair cells themselves are certainly involved<sup>3</sup>. However, in at least one auditory organ, the turtle cochlea, frequency tuning is greatly enhanced by the electrical properties of the hair-cell membrane; each cell in this organ behaves as an electrical resonator tuned to a narrow band of frequencies<sup>4</sup>. Using the 'Gigaseal', whole-cell recording technique<sup>5</sup>, we have investigated the biophysical basis of similar resonant behaviour in enzymatically isolated hair cells from the bullfrog sacculus. We report here the identification of three voltage- and ion-dependent conductances which may contribute to the electrical tuning mechanism: a non-inactivating calcium conductance, an A-type  $\text{K}^+$  conductance, and a  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  conductance<sup>6</sup>.

Hair cells were dissociated from the excised sacculus of the bullfrog and whole-cell recording was performed as described in Fig. 1 legend. Damped oscillations in membrane potential

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can be evoked in isolated cells by the injection of a square step of depolarizing current (Fig. 2). As for hair cells in the turtle cochlea<sup>4</sup>, the oscillation frequency is a function of the sign and magnitude of the injected current, increasing with depolarization. However, the oscillation frequency at resting potential, after the termination of the current step, is relatively independent of the value of the preceding current injection<sup>4</sup>. All 20 cells we stimulated in this way oscillated at frequencies ranging from 80 to 160 Hz at resting potentials of  $-58$  to  $-65$  mV. This resonant behaviour does not require the presence of the hair bundle, as occasional cells that had lost their hair bundles during the isolation procedure showed similar oscillations. In addition, oscillations of similar frequency were found by using conventional microelectrode recording techniques on undissociated cells with or without hair bundles; the oscillations are therefore not an artefact of the cell isolation procedure.

Depolarization of hair cells in voltage-clamp experiments elicited several specific ionic currents. Some currents were not characterized because they could be activated only outside the voltage range of oscillations or in only a small proportion ( $<10\%$ ) of the cells. However, one inward and two outward currents were observed at voltages near the resting potential in more than 90% of the cells studied. These three currents were isolated and are described below.

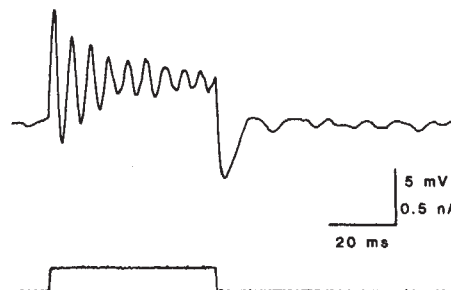
The substitution of  $\text{Cs}^+$  for  $\text{K}^+$  inside the pipette blocked most of the depolarization-induced outward current, leaving a sustained inward current (Fig. 3A). We have identified this as a  $\text{Ca}^{2+}$  current based on the characteristics that it has in common with the  $\text{Ca}^{2+}$  currents of other preparations<sup>7</sup>. It is carried (in order of decreasing effectiveness) by  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ca}^{2+}$ . Also, it is blocked (in order of decreasing effectiveness) by  $\text{Cd}^{2+}$  and  $\text{Ni}^{2+}$ , and is abolished by removal of external  $\text{Ca}^{2+}$  with or without  $\text{Mg}^{2+}$  substitution.

The  $\text{Ca}^{2+}$  current is activated at potentials more positive than  $-60$  to  $-55$  mV. At  $-30$  mV, the time course of its activation is approximated well by a third-order Hodgkin-Huxley kinetic scheme<sup>8</sup> with a time constant of 0.4 ms. A contaminating outward current, insensitive to internal  $\text{Cs}^+$  and external tetraethylammonium ion (TEA), 4-aminopyridine (4-AP), or  $\text{Ba}^{2+}$ , precluded kinetic analysis above  $-30$  mV. Our initial observations of the hair cell's  $\text{Ca}^{2+}$  current suggested that, unlike those in other preparations<sup>7</sup>, it does not show inactivation (see Fig. 3A). In order not to obscure any possible  $\text{Ca}^{2+}$ -dependent inactivation<sup>9</sup>, we measured the inward current using an internal solution that contained  $\text{Ca}^{2+}$  buffered to  $10^{-8}$  M with 0.1 mM EGTA, a level of buffer that should not significantly affect the cell's endogenous  $\text{Ca}^{2+}$  buffering capacity. In three cells tested, the inward currents produced during 1-s voltage steps ranging from  $-50$  to  $-42$  mV did not decline from their maximal values of  $-20$  to  $-90$  pA. Thus, at least at these levels of activation, the hair cell's  $\text{Ca}^{2+}$  conductance is capable of sustained activity.

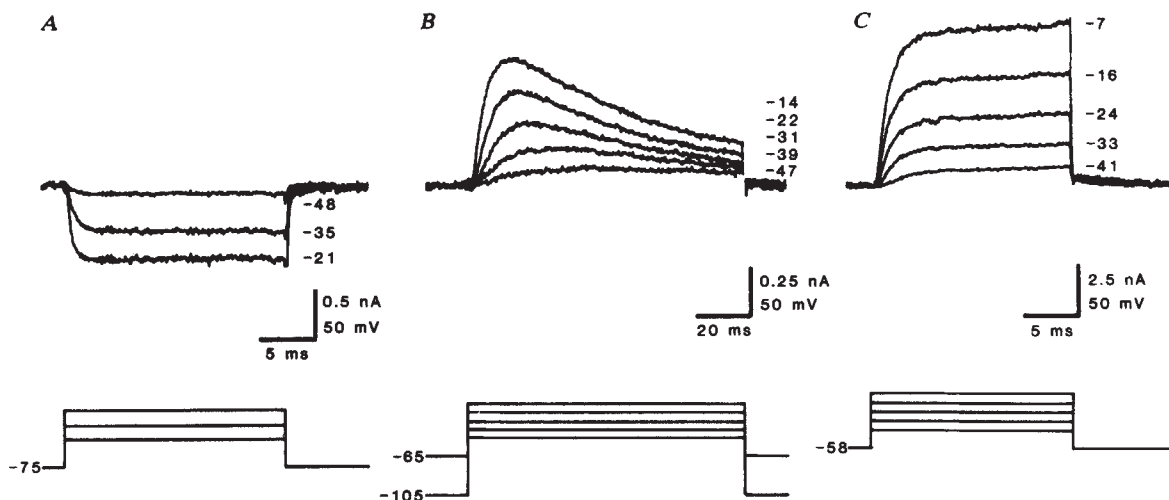
Two outward  $\text{K}^+$  currents are produced by depolarization of hair cells dialysed with  $\text{K}^+$  internal solution. We consider one of these, a transient  $\text{K}^+$  current, to be an A-type  $\text{K}^+$  current based on similarities to the A currents described in molluscan neurones<sup>10,11</sup>. Two of these properties, steady-state voltage-dependent inactivation and TEA insensitivity, were used in the experiment of Fig. 3B to isolate the hair cell's A current from the  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents. This transient current is the only one we have observed routinely in hair cells that shows voltage-dependent inactivation. It reverses at  $-90$  mV, close to the calculated  $\text{K}^+$  Nernst potential of  $-104$  mV. The A current was never observed when  $\text{Cs}^+$  was substituted for internal  $\text{K}^+$ . Like that of molluscan neurones, the hair cell's A current is relatively insensitive to external TEA<sup>12</sup>, but is blocked in a voltage-dependent manner by 20 mM 4-AP<sup>13</sup>. In contrast to the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current, it is not abolished by divalent cations that block the  $\text{Ca}^{2+}$  current<sup>12</sup>. It becomes activated at voltages more positive than  $\sim -50$  mV, and its kinetics can be fitted well by a Hodgkin-Huxley  $m^3h$  scheme<sup>8</sup> in which the time constant of activation is 10 ms and that of inactivation is 80 ms at  $-40$  mV.



**Fig. 1** Differential-interference-contrast light micrograph of a hair cell, isolated from bullfrog sacculus, in contact with a recording pipette. Sacculi were dissected from the animal and treated with  $0.5 \text{ mg ml}^{-1}$  papain (Calbiochem) in a solution containing (in mM):  $\text{Na}^+$  120,  $\text{K}^+$  2,  $\text{Ca}^{2+}$  0.1, L-cysteine 2.5,  $\text{Cl}^-$  120, D-glucose 3 and HEPES 5 (pH 7.2), for 25 min at  $22^\circ\text{C}$ . The organs were then washed with a similar solution lacking papain and cysteine and containing  $0.5 \text{ mg ml}^{-1}$  bovine serum albumin, and were scraped gently with a sharpened tungsten needle to free individual hair cells. After isolation, single cells readily formed seals of 1–20 G $\Omega$  shunt resistance with the tips of flint-glass pipettes containing (in mM): 126  $\text{K}^+$  or  $\text{Cs}^+$ ,  $\text{Ca}^{2+}$  1,  $\text{Mg}^{2+}$  2, aspartate 120,  $\text{Cl}^-$  6, D-glucose 3, EGTA 2 and HEPES 5 (pH 7.2), with  $10^{-7}$  M free  $\text{Ca}^{2+}$ . Slight suction was applied to rupture the patch of membrane under the pipette, and the cells were current- or voltage-clamped through the pipette<sup>5</sup>. The external solution normally used during recording contained (in mM):  $\text{Na}^+$  120,  $\text{K}^+$  2,  $\text{Ca}^{2+}$  4,  $\text{Cl}^-$  128, D-glucose 3 and HEPES 5 (pH 7.2). In some experiments, TEA or 4-AP was substituted for an equal amount of external NaCl, or different divalent cations were substituted for external  $\text{Ca}^{2+}$ . External solutions were changed rapidly by lifting cells from the bottom of the chamber and passing them sequentially through different solutions flowing continuously from four 0.5-mm diameter tubes<sup>24</sup>. All experiments were done at  $20$ – $22^\circ\text{C}$ . Scale bar, 10  $\mu\text{m}$ .



**Fig. 2** Voltage oscillations in an isolated hair cell. The upper trace is a voltage record obtained by clamping the cell's membrane current to the value shown in the lower trace. Data were digitized at an interval of  $256 \mu\text{s}$  per point with a PDP 11/34 computer. During the current step the frequency of voltage oscillations was  $\sim 180$  Hz, while at the resting potential ( $-55$  mV) it was about 90 Hz. The latter value was measured by averaging the cell's responses to eight stimuli of different sizes, then taking the mean frequency of the first five averaged oscillations after the current step. A single response is shown.



**Fig. 3** Voltage-clamp recordings from isolated hair cells, showing the three ionic currents. In each panel the upper set of traces shows membrane current and the lower set represents command voltage. To the right of each current trace is given the corresponding command voltage in mV; the holding potential in mV is shown to the left of the voltage command traces in each panel. For *A* and *C*, voltage steps were presented every 400 ms, and the responses were sampled at an interval of 64  $\mu$ s per point and averaged; for *B*, the respective values were 1 s and 256  $\mu$ s per point. Data were corrected for capacitive and leakage currents by subtracting from each current trace an appropriate multiple of the passive current elicited by a small depolarizing voltage step. *A*,  $\text{Ca}^{2+}$  current. In this experiment 126 mM  $\text{Cs}^+$  replaced all  $\text{K}^+$  in the pipette. These data were recorded after a steady-state block of outward current by internal  $\text{Cs}^+$  was obtained,  $\sim 30$  s after the membrane patch under the pipette was ruptured. Means of five presentations are shown. *B*, Transient  $\text{K}^+$  current (*A* current). These data were obtained by the following subtraction method. With 20 mM external TEA blocking the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current, a series of voltage steps was applied to the cell from holding potentials of  $-65$  or  $-105$  mV. At  $-65$  mV,  $\sim 95\%$  of the *A* current was inactivated, and the voltage steps elicited primarily  $\text{Ca}^{2+}$  current. At  $-105$  mV the *A* current's inactivation was removed, and the voltage steps elicited the sum of  $\text{Ca}^{2+}$  and *A* currents. Subtracting the former set of currents from the latter isolated the *A* current from the  $\text{Ca}^{2+}$  current. Similar but noisier results were obtained without TEA. Means of four presentations. *C*,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current. The responses shown were obtained by subtracting the currents produced by voltage steps in the presence of 20 mM TEA (a fully blocking dose) from those produced in its absence. Means of two presentations. The series resistance contributed by the pipettes was typically about 4 M $\Omega$  after cell rupture; this was determined in current-clamp conditions by measuring the nearly instantaneous voltage change produced by a step of current. The voltage error introduced in voltage-clamp experiments by current flowing across the series resistance was compensated for by adding to the command voltage a correction signal proportional to membrane current. The data shown here were obtained from three different cells.

The largest current produced by depolarization of the hair cell is a  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current (Fig. 3C). It was isolated by using its sensitivity to TEA, which specifically blocks the  $\text{Ca}^{2+}$ -dependent outward current in these cells with a 50% blocking concentration of 1 mM. The data in Fig. 3C were obtained by subtracting the responses to voltage steps in the presence of 20 mM TEA from those in its absence. The resultant difference current reverses at  $-82$  mV and is blocked by internal  $\text{Cs}^+$ . The following observations suggest that this current is  $\text{Ca}^{2+}$ -dependent. First, all of the TEA-blockable outward current in single cells was abolished by external agents or conditions that block the hair cell's  $\text{Ca}^{2+}$  current:  $\text{Ni}^{2+}$ ,  $\text{Cd}^{2+}$ , replacement of  $\text{Ca}^{2+}$  by  $\text{Mg}^{2+}$ , or removal of external  $\text{Ca}^{2+}$  without substitution. Like the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents of other preparations<sup>14</sup>, it can also be eliminated by  $\text{Ba}^{2+}$  (but not  $\text{Sr}^{2+}$ ) substitution for external  $\text{Ca}^{2+}$  but is relatively insensitive to 4-AP (20 mM blocks  $<5\%$  of the current). Lowering the external  $\text{Ca}^{2+}$  concentration from 4 to 0.5 mM (with  $\text{Mg}^{2+}$  substitution) slows the kinetics and lowers the steady-state magnitude of this current. Finally, the cell's current-voltage relationship measured 20 ms after the start of a voltage-clamp step peaked at  $+30$  to  $+40$  mV, producing an 'N'-shaped curve similar to that found for other preparations having a  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  conductance<sup>14</sup>. The hair cell's  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current is activated at potentials above  $-60$  to  $-45$  mV; its kinetic behaviour cannot be fitted well by a Hodgkin-Huxley scheme<sup>8</sup> of any order.

The currents identified in this study account for some of the previously reported physiological properties of hair cells. The  $\text{Ca}^{2+}$  conductance we have observed is probably involved in the  $\text{Ca}^{2+}$ -dependent<sup>15</sup> release of transmitter from hair cells onto afferent nerve fibres: its activation at voltages close to the resting potential and its failure to be significantly inactivated are consistent with a role in the tonic release of transmitter by hair cells at rest<sup>16</sup>. The *A* current and particularly the large  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current are probably responsible for the striking

outward rectification observed in hair cells of the bullfrog sacculus<sup>17</sup> and the turtle cochlea<sup>18</sup>.

Which of the ionic currents in the hair cell contribute(s) to membrane-potential oscillations? Because oscillations persist in isolated cells lacking hair bundles and efferent nerve terminals, modulation of either the transduction<sup>17</sup> or the postsynaptic efferent<sup>19</sup> conductance is not essential for the oscillation phenomenon. Similarly, as the *A* current is largely inactivated at resting potential, it may not be significantly involved. The best candidates for currents underlying the hair cell's electrical resonance are the  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents. Apart from those discussed above, these two currents are the only ones we have routinely observed that are active in the voltage range in which oscillations occur. Moreover, TEA, a rather specific blocker of the hair cell's  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current, effectively reduces or abolishes oscillations, a result also obtained with turtle hair cells<sup>4,18</sup>.

It has been shown in several preparations that electrical resonance can be produced by the interactions of relatively fast, voltage-dependent conductances<sup>20-22</sup>. In those and other cell types, the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current typically shows rather slow kinetic behaviour, and is thought to modulate membrane potential slowly, leading in some cases to 'bursting' patterns of activity<sup>14,21,22</sup>. In this regard, it is interesting that the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  conductance in hair cells is activated 10–100 times more rapidly for a given range of depolarizations than any that have been reported previously (but see ref. 23). This rapid kinetic behaviour may allow the hair cell's  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel to participate in electrical processes usually reserved for fast, voltage-dependent channels.

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- Russell, I. J. & Sellick, P. M. *J. Physiol., Lond.* **284**, 261–290 (1978).
- Dallos, P., Santos-Sacchi, J. & Flock, A. *Science* **218**, 582–584 (1982).
- Dallos, P. A. *Rev. Psychol.* **32**, 153–190 (1981).
- Crawford, A. C. & Fettiplace, R. *J. Physiol., Lond.* **312**, 377–412 (1981).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Lewis, R. S. *Soc. Neurosci. Abstr.* **8**, 728 (1982).
- Hagiwara, S. & Byerly, L. A. *Rev. Neurosci.* **4**, 69–125 (1981).
- Hodgkin, A. L. & Huxley, A. F. *J. Physiol., Lond.* **117**, 500–544 (1952).
- Eckert, R. & Tillotson, D. L. *J. Physiol., Lond.* **314**, 265–280 (1981).
- Connor, J. A. & Stevens, C. F. *J. Physiol., Lond.* **213**, 21–30 (1971).
- Neher, E. *J. gen. Physiol.* **58**, 36–53 (1971).
- Thompson, S. H. *J. Physiol., Lond.* **265**, 465–488 (1977).
- Thompson, S. J. *gen. Physiol.* **80**, 1–18 (1982).
- Meech, R. W. A. *Rev. Biophys. Bioengng* **7**, 1–18 (1978).
- Furukawa, T. & Matsuura, S. *J. Physiol., Lond.* **276**, 193–209 (1978).
- Sand, O., Ozawa, S. & Hagiwara, S. *J. comp. Physiol.* **102**, 13–26 (1975).
- Corey, D. P. & Hudspeth, A. J. *Nature* **281**, 675–677 (1979).
- Crawford, A. C. & Fettiplace, R. *J. Physiol., Lond.* **315**, 317–338 (1981).
- Art, J. J., Crawford, A. C., Fettiplace, R. & Fuchs, P. A. *Proc. R. Soc. B* **216**, 377–384 (1982).
- Mauro, A., Conti, F., Dodge, F. & Schor, R. *J. gen. Physiol.* **55**, 497–523 (1970).
- Morris, C. & Lecar, H. *Biophys. J.* **35**, 193–213 (1981).
- Plant, R. E. *Biophys. J.* **21**, 217–237 (1978).
- Adams, P. R., Constanti, A., Brown, D. A. & Clark, R. B. *Nature* **296**, 746–749 (1982).
- Yellen, G. *Nature* **296**, 357–359 (1982).

## Inhibition of pathogenic effect of effector T cells by specific suppressor T cells during influenza virus infection in mice

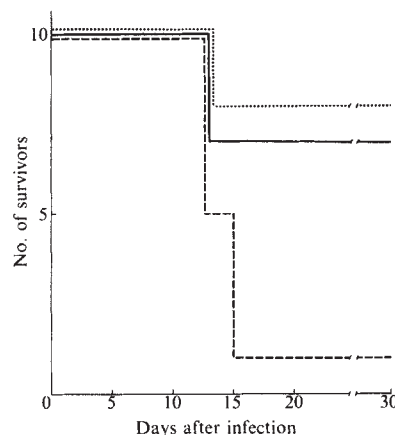
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Mice infected with an aerosol of influenza type A virus, or immunized with purified UV-inactivated whole virus or with viral subunits, develop a transient delayed-type hypersensitivity (DTH) which peaks 5–7 days after immunization<sup>1</sup>. The intensity of DTH is greatly enhanced and sustained when mice are pretreated with cyclophosphamide<sup>1</sup>. The reaction is maximal 24 h after elicitation, has classical tuberculin-type histology and is transferable by immune H-2I region restricted Lyt-1<sup>+</sup>2<sup>+</sup> T cells (T<sub>d</sub>) but not by immune serum<sup>1,2</sup>. These T<sub>d</sub> cells not only fail to protect mice against influenza virus infection<sup>3</sup>, but increase the mortality rate due to influenzal pneumonia following challenge with homologous lethal virus<sup>3,4</sup>. On the other hand, antigen-specific suppressor T (T<sub>s</sub>) cells which inhibit DTH are readily generated during influenza virus infection, and are detectable for at least 40 days thereafter<sup>5</sup>. The ease with which they are induced and maintained during the infection may be of evolutionary advantage<sup>6</sup>. In support of this, we now report that these T<sub>s</sub> cells can reverse the pathogenic effect of T<sub>d</sub> cells thereby demonstrating a beneficial influence of T<sub>s</sub> cells in a viral disease.

Splenic T cells were obtained from inbred CBA/T6T6 mice injected intraperitoneally (i.p.) with 10 µg of purified UV-inactivated PR8 virus 3 weeks previously and cultured *in vitro* for 5 days with PR8 virus-infected CBA spleen cells as described by Leung and Ada<sup>3</sup>. These secondary T<sub>d</sub> cells had minimal cytotoxic activity but transferred PR8 specific DTH both locally and systemically (data not shown). T<sub>s</sub> cells were obtained from the spleens of CBA mice infected 2 weeks earlier with a sublethal dose of PR8 virus by aerosol as described previously<sup>5</sup>. Normal T cells (T<sub>n</sub>) were collected from the spleens of unprimed CBA mice. All T-cell preparations were enriched by an immunoglobulin-rosetting method described by Parish *et al.*<sup>7</sup>. The T<sub>s</sub> cells have been extensively characterized<sup>5</sup> in that they are Lyt-1<sup>+</sup>2<sup>+</sup>Ia<sup>+</sup>, inhibit the induction of DTH and recognize only the subtype-specific epitopes of the haemagglutinin.

To demonstrate the effect of T<sub>s</sub> on T<sub>d</sub> cells, primary T<sub>d</sub> cells were co-cultured for 5 days with T<sub>s</sub> cells or T<sub>n</sub> cells at a ratio



**Fig. 1** Reversal of the pathogenic effect of T<sub>d</sub> cells by T<sub>s</sub> cells during influenza virus infection in mice. CBA/T6T6 mice age 10–12 weeks of either sex were used throughout the experiments. Primary T<sub>d</sub> cells were obtained from spleens of mice injected 3 weeks earlier with 10 µg of purified UV-inactivated PR8 virus<sup>1</sup> (A/Puerto Rico/8/34, H<sub>1</sub>N<sub>1</sub>). T<sub>s</sub> cells were collected from the spleens of mice infected 2–3 weeks earlier with an aerosol of PR8 virus (1/4 of LD<sub>50</sub> dose). T<sub>n</sub> cells were from normal spleens. All T cells were enriched from the spleen cell preparation by a single step immunoglobulin-rosetting technique<sup>7</sup>. 2.5 × 10<sup>8</sup> primary T<sub>d</sub> cells were co-cultured with 1.2 × 10<sup>8</sup> T<sub>s</sub> cells or T<sub>n</sub> cells in 100 ml culture medium (RPMI 1640 (Gibco), with 10% fetal calf serum and 10<sup>-5</sup> M 2-mercaptoethanol) together with 2 × 10<sup>7</sup> normal spleen cells which had been infected *in vitro* with PR8 virus. The cultures in standing 75 cm<sup>2</sup> Falcon flasks were kept at 37 °C in 5% CO<sub>2</sub> for 5 days. Viable cells (25–35% of input) were collected and injected i.v. (3 × 10<sup>7</sup> cells per mouse) into syngeneic recipients which had been infected 24 h earlier with an aerosol of one LD<sub>50</sub> of PR8 virus. Mortality was observed and recorded for 28 days. (—) T<sub>n</sub> alone, (---) T<sub>n</sub> + T<sub>d</sub>, (···) T<sub>s</sub> + T<sub>d</sub>.

of 2:1 in the presence of PR8 virus-infected syngeneic spleen cells. At the end of the culture period, viable T cells were collected and transferred intravenously (i.v.) into CBA mice which had been infected 24 h earlier with an aerosol of approximately one LD<sub>50</sub> PR8 virus. When mice were injected with 3 × 10<sup>7</sup> T<sub>n</sub> cells alone, 3 out of 10 died by day 28 (Fig. 1). This figure rose to 9 out of 10 when they were injected with the same number of viable cells from T<sub>d</sub> + T<sub>n</sub> culture. However, if the T<sub>d</sub> cells were cultured in the presence of T<sub>s</sub> cells, only 2 out of 10 of the recipients died. Mice at the terminal stage of infection showed extensive lung consolidation consistent with the pathology of influenzal pneumonia<sup>3,8</sup>.

Although the T<sub>s</sub>-cell preparation was essentially free of B cells, it contained also cytotoxic T (T<sub>c</sub>) cells and helper T (T<sub>h</sub>) cells for antibody production<sup>5</sup>. Thus it can be argued that the protective effect seen was due to T<sub>c</sub> cells which eliminated the antigen-presenting cells thereby removing the antigenic signals necessary for driving the primary T<sub>d</sub> cells to mature secondary T<sub>d</sub> cells. It is also possible that the protection conferred by the T<sub>s</sub>-cell preparation was due to T<sub>h</sub> cells which on transference accelerated and augmented humoral antibody production in the recipients. These possibilities were tested in the following experiment.

The T<sub>s</sub>-cell preparation was treated *in vitro* with various antisera and complement as described in the legend to Table 1. After washing, they were co-cultured with T<sub>d</sub> cells and transferred to PR8 infected mice. In this experiment, 6 out of 10 (60%) infected mice died by day 28 when injected with control T<sub>n</sub> cells (Table 1). This figure rose to 8 out of 8 (100%) when the mice were injected with T<sub>d</sub> cells co-cultured with T<sub>n</sub> cells. This was reversed to 4 out of 8 (50%) when the T<sub>d</sub> cells were co-cultured with an untreated T<sub>s</sub>-cell preparation. However, when the T<sub>s</sub>-cell preparation was treated with anti-Thy-1.2 + C' or anti-Lyt-1.1 + C', the protective effect of the

**Table 1** Effect of antiserum and complement on  $T_s$  cells

| Group | Cells transferred<br>( $3 \times 10^7$ i.v.) | Mortality<br>(%) | Cytotoxic T-cell<br>activities (%) |      | Delayed-type hypersensitivity          |                      | Serum<br>antibody |
|-------|----------------------------------------------|------------------|------------------------------------|------|----------------------------------------|----------------------|-------------------|
|       |                                              |                  | 25:1                               | 50:1 | Specific DTH<br>( $\times 10^{-2}$ mm) | % Suppression<br>(P) |                   |
| A     | Td+Tn                                        | 8/8 (100)        | 0                                  | 2    | 25 $\pm$ 0                             | 0                    | <10               |
| B     | Td+Ts                                        | 4/8 (50)         | 35                                 | 50   | 9 $\pm$ 3                              | 64 (<0.005)          | <10               |
| C     | Td+Ts $\bar{a}$ Thy-1.2                      | 8/8 (100)        | 0                                  | 1    | 23 $\pm$ 2                             | 8 (NS)               | <10               |
| D     | Td+Ts $\bar{a}$ Lyt-1.1                      | 7/8 (87)         | 40                                 | 51   | 23 $\pm$ 5                             | 8 (NS)               | <10               |
| E     | Td+Ts $\bar{a}$ Lyt-2.1                      | 3/8 (37)         | 4                                  | 10   | 11 $\pm$ 3                             | 56 (<0.01)           | <10               |
| F     | Tn                                           | 6/10 (60)        | 0                                  | 3    | 0                                      | —                    | <10               |

$T_s$  cells were prepared as described in Fig. 1 legend. Before co-culturing with primary  $T_d$  cells,  $T_s$  cells were treated *in vitro* (at  $5 \times 10^7$  per ml) with the following sera and complement (Cedarlane) in a two-step method: Anti-Thy-1.2 (F7D5, monoclonal IgM; Olac 1976 Ltd) 1/500; anti-Lyt-1.1 (monoclonal IgG2b, NEI-003, NEN), 1/300; anti-Lyt-2.1 (monoclonal IgG2b, NEI-004, NEN) 1/300. The treated cells were washed thoroughly and added to the culture as described in Fig. 1 legend. At the end of 5 days culture, viable cells were collected and transferred ( $3 \times 10^7$  per recipient) i.v. into 24 h pre-infected CBA mice and the effect on mortality observed. A sample of the cells was tested for cytotoxicity or transference of DTH as follows: cytotoxicity assay, target cells were  $^{51}\text{Cr}$ -labelled PR8 or B/Lee virus infected L-929 cells or P815 cells. Assay in triplicate was set up in 96 wells round-bottom Linbro plates at  $10^4$  target cells per 200  $\mu\text{l}$  medium per well. The plates were incubated at 37 °C in 5%  $\text{CO}_2$  for 4 h and the  $^{51}\text{Cr}$  released counted in a gamma counter. Spontaneous release (medium only) was <10% of total lysis by 1% NP40. Results were expressed as % specific  $^{51}\text{Cr}$  release at effector:target ratio of 25:1 or 50:1 against PR8-infected L929 cells. Specific cytolytic activity against B/Lee virus or PR8 virus infected P815 cells was negligible. DTH transfer,  $5 \times 10^6$  viable cells together with 10  $\mu\text{g}$  purified UV-inactivated PR8 virus were injected into the right hind footpad of normal CBA mice. An equal volume (50  $\mu\text{l}$ ) of medium was injected into the left hind footpad. Footpad swelling was measured 24 and 48 h after injection. DTH was expressed as the difference in thickness of right versus left hind footpad in  $10^{-2}$  mm  $\pm$  s.e. Specific DTH denotes value after subtracting background reaction due to transference of  $T_n$ +PR8 virus (group F). The % suppression was calculated by comparing with group A,  $n=5$ . Two days after cell transfer, a blood sample (100  $\mu\text{l}$ ) was taken from each mouse and anti-PR8 antibodies in the serum were estimated in a solid phase radioimmunoassay. Flexiplates were coated with purified UV-inactivated PR8 virus and reacted with dilutions of the sera. The assay in duplicate was developed with  $^{125}\text{I}$ -labelled, affinity purified rabbit anti-mouse IgG. End point was taken as the highest dilution with  $>2 \times \text{c.p.m.}$  of the corresponding control normal serum.

**Table 2** Analysis of specific antibody in mice infected with PR8 virus

| Cells transferred<br>( $3 \times 10^7$ i.v.) | Mortality<br>(%) | Serum anti-PR8 antibody titre (day 7) |     |      |       |       |       |
|----------------------------------------------|------------------|---------------------------------------|-----|------|-------|-------|-------|
|                                              |                  | IgA                                   | IgM | IgG1 | IgG2a | IgG2b | IgG3  |
| $T_d+T_n$                                    | 12/12 (100)      | <10                                   | <10 | 320  | 640   | 320   | 640   |
| $T_d+T_s$                                    | 5/12 (42)        | <10                                   | <10 | 640  | 640   | 640   | 640   |
| $T_d+T_s \bar{a}$ Lyt-1.1                    | 12/12 (100)      | <10                                   | <10 | 640  | 640   | 640   | 640   |
| $T_d+T_s \bar{a}$ Lyt-2.1                    | 5/12 (42)        | <10                                   | <10 | 640  | 640   | 1,280 | 1,280 |
| $T_n$                                        | 7/12 (58)        | <10                                   | <10 | 80   | 80    | 160   | 80    |

$T_d$  and  $T_s$  cells were prepared, treated, incubated and transferred exactly as described in legends to Fig. 1 and Table 1. Mice were also infected as described. Serum samples were obtained 7 days after infection. They were pooled for each group and titrated by solid-phase radioimmunoassay as follows: Dynatech U-bottom flexiplates were coated with purified UV-inactivated PR8 virus and reacted with dilution of the sera. The plates were washed, and 50  $\mu\text{l}$  of a 1/100 dilution of rabbit anti-mouse immunoglobulin class antiserum (Miles) were added. The assay in duplicate was developed with  $^{125}\text{I}$ -labelled goat anti-rabbit antibody (affinity chromatography purified IgG fraction). End point was taken as the highest dilution with  $>2 \times \text{c.p.m.}$  of the corresponding control normal serum.

$T_s$ -cell preparation was completely abrogated, the mortality rose to 8 out of 8 (100%) and 7 out of 8 (78%) respectively. In contrast, treatment with anti-Lyt-2.1 serum and C' had little or no effect on the ability of the  $T_s$ -cell preparation to ablate the detrimental influence of  $T_d$  cells (3 out of 8, 37%).

Thus it seems that the protective effect of the  $T_s$ -cell preparation is exerted by a population of Thy-1<sup>+</sup>, Lyt-1<sup>+</sup>2<sup>+</sup> cells. The cytotoxic activity of the cell suspension does not appear to have a role, as treatment with anti-Lyt-2.1 serum and complement almost totally abrogated the cytotoxic activity of the  $T_s$ -cell preparation, yet left intact their ability to inhibit the pathogenic effect of  $T_d$  cells (Table 1). Conversely, treatment with anti-Lyt-1.1 serum and complement completely abolished the suppression of  $T_d$  activity, yet had no effect on the cytotoxic reaction (Table 1). The ability of  $T_d$  cells to transfer DTH locally was also strongly suppressed by the  $T_s$ -cell preparation. Such suppression was abolished by treatment with anti-Thy-1.2 or anti-Lyt-1.1 and complement but not with anti-Lyt-2.1 and complement (Table 1). Furthermore, there was no detectable serum anti-PR8 antibody 3 days after infection in any of the groups. The early time point was chosen as this would facilitate the detection of (1) any passive transfer of antibody due to residual plasma cells, and (2) the accelerated emergence of antibody.

The possibility that isotype-specific  $T_h$  cells may be involved was also investigated. Mice treated similarly as in the experiment depicted in Table 1 were bled 7 days after infection. By this time antibodies were detected in all groups of mice. Mice injected with  $T_d+T_n$  cells produced significantly higher levels of serum IgG antibodies than did control mice injected with  $T_n$  cells alone (Table 2). This effect is clearly due to  $T_h$  cells present in the  $T_d$ -cell preparation. However, the addition of treated or untreated  $T_s$  cells to  $T_d$  cells did not quantitatively or qualitatively modify the subsequent serum antibody production, yet, at the same time, significantly altered the outcome of the infection of the recipients (Table 2). Thus it seems that the beneficial effect of the  $T_s$  cell preparation is not likely to be due to the presence of  $T_h$  cells for antibody synthesis, since such help has already been provided to an optimal level by  $T_h$  cells in the  $T_d$  cell preparation. Table 3 summarizes the effect of  $T_d$  and  $T_s$  cells on the outcome of LD<sub>50</sub> PR8 virus infection. It is clear that group A is significantly different from group F and that groups B and E are significantly different from group A but not group F. Conversely groups C and D are significantly different from group F but not group A.

From these results, it appears that the abrogation of the pathogenic influence of  $T_d$  cells is mediated by Lyt-1<sup>+</sup>2<sup>+</sup> $T_s$  cells



**Table 3** Effect of  $T_d$  and  $T_s$  cells on the survival of LD50 PR8 virus infected mice

| Group | Cells transferred           | Accumulated*<br>mortality | P values†<br>compared with |         |
|-------|-----------------------------|---------------------------|----------------------------|---------|
|       |                             |                           | Group A                    | Group F |
| A     | $T_d + T_n$                 | 29/30                     | —                          | <0.001  |
| B     | $T_d + T_s$                 | 11/30                     | <0.001                     | NS      |
| C     | $T_d + T_s \bar{a}$ Thy-1.2 | 8/8                       | NS                         | <0.05   |
| D     | $T_d T_s \bar{a}$ Lyt-1.1   | 19/20                     | NS                         | <0.001  |
| E     | $T_d + T_s \bar{a}$ Lyt-2.1 | 8/20                      | <0.001                     | NS      |
| F     | $T_n$                       | 16/32                     | <0.001                     | —       |

\* Data pooled from Fig. 1, Tables 1 and 2.

† Fisher's exact (2-tail) for  $2 \times 2$  contingency tables; NS, not significant.

which are probably analogous to the cells suppressing the cutaneous DTH reaction, but distinct from either  $T_h$  or  $T_c$  cells. The Lyt phenotype of these  $T_s$  cells is in agreement with that of T cells which suppress in other DTH systems<sup>9-12</sup>. They suppress the induction of  $T_d$  cells but have little or no effect on the expression of DTH<sup>5</sup> and do not influence the antibody response. In the present system,  $T_s$  cells co-transferred with the secondary  $T_d$  cells into PR8 virus-infected mice failed to inhibit the pathogenic effect of  $T_d$  cells (data not shown). Thus, the  $T_s$  cells reported here may be analogous to the  $T_{s1}$  cells described elsewhere<sup>13,14</sup>. The mechanism by which the  $T_s$  cells inhibit the  $T_d$  effector cells remains unclear.

One of the basic characteristics of DTH is its specific induction but nonspecific manifestation. However, by arming the host macrophages nonspecifically, the DTH response may produce severe host tissue damage, particularly if infections occur in vital organs. It makes biological sense, therefore, that DTH reactions be kept in check and hence allow the survival of the host. It is not surprising that the DTH reaction has turned out to be tightly regulated by  $T_s$  cells.

The results reported here, as far as we are aware, show directly for the first time the beneficial effect of specific  $T_s$  cells against a potentially lethal viral infection. A similar phenomenon has been seen in mice chronically infected with *Schistosoma mansoni*<sup>15</sup>, where adoptively transferred  $T_s$  cells reduced granuloma formation, a DTH-related response. However, DTH responses are nevertheless vital to the host in systems where the primary target of the infectious organism is the macrophage. In this situation the generation of  $T_s$  cells for DTH could greatly compromise survival of the host. For example, death from *Leishmania tropica* infection in genetically susceptible mice, is attributable to the generation of antigen-specific  $T_s$  cells for DTH<sup>12,16</sup>. Thus the *in vivo* capacity of DTH suppressor cells in both adversely and beneficially influencing the outcome of experimental infection is now firmly established.

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- Liew, F. Y., Russell, S. M. & Brand, C. M. *Eur. J. Immunol.* **9**, 783 (1979).
- Leung, K. N., Ada, G. L. & McKenzie, I. F. C. *J. exp. Med.* **151**, 815 (1980).
- Leung, K. N. & Ada, G. L. *Scand. J. Immunol.* **12**, 393 (1980).
- Cate, T. R. & Mold, N. G. *Inf. Immun.* **11**, 908 (1975).
- Liew, F. Y. & Russell, S. M. *J. exp. Med.* **151**, 799 (1980).
- Liew, F. Y. *Immun. Today* **3**, 18 (1982).
- Parish, C. R., Kirov, S. M., Bowers, N. A. & Blanden, R. V. *Eur. J. Immunol.* **4**, 808 (1974).
- Wagner, R. R. *Yale J. Biol. Med.* **28**, 598 (1956).
- Ramshaw, I. A., McKenzie, I. F. C., Bretcher, P. A. & Parish, C. R. *Cell Immun.* **31**, 364 (1977).
- Liew, F. Y., Sia, D. Y., Parish, C. R. & McKenzie, I. F. C. *Eur. J. Immunol.* **10**, 305 (1980).
- Kina, T., Nishikawa, S. & Katsura, Y. *Immunology* **47**, 525 (1982).
- Liew, F. Y., Hale, C. & Howard, J. G. *J. Immunol.* **128**, 1917 (1982).
- Germain, R. N. & Benacerraf, B. *Scand. J. Immunol.* **13**, 1 (1981).
- Okuda, K., Minami, M., Furusawa, S. & Dorf, M. E. *J. exp. Med.* **154**, 1838 (1981).
- Green, W. F. & Colley, D. G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1152 (1981).
- Howard, J. G., Hale, C. & Liew, F. Y. *J. exp. Med.* **153**, 557 (1981).

## Task variables determine which biological clock controls circadian rhythms in human performance

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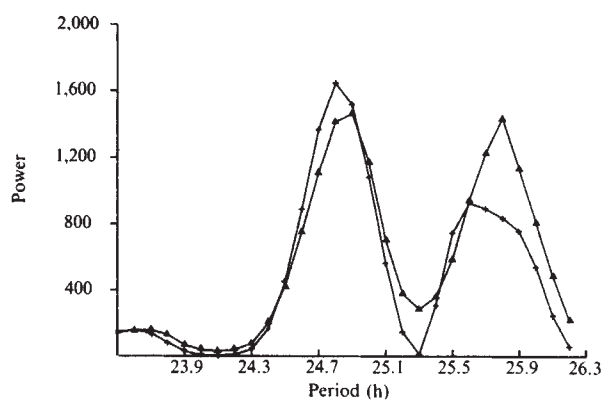
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There are circadian (~24 h) rhythms for a wide range of human physiological and psychological functions including mood and performance efficiency<sup>1</sup>. These rhythms are self-sustaining in conditions of temporal isolation, indicating that internal oscillators (or biological clocks) control them<sup>2</sup>. Recent research has proposed an endogenous two-oscillator model of the human circadian system, with one oscillator indicated by the core body temperature rhythm and a second oscillator responsible for the daily cycle of sleep and wakefulness<sup>3,4</sup>. The present study was designed to produce a desynchronization in period between the two oscillators, to assess directly the behaviour of the rhythms of different performance tasks. The results, reported here, indicate that a simple manual dexterity task is almost entirely under the control of the temperature rhythm oscillator, whereas a more complex cognitive task demonstrates a periodicity which appears to be influenced by those oscillators controlling temperature and the sleep/wake cycle.

Previously, the correlation between circadian performance rhythms and the body temperature rhythm has been much emphasized<sup>5</sup>. However, recent research has demonstrated that the phase of a circadian performance rhythm is dependent to a major degree on the type of task being performed. Simple repetitive perceptual-motor tasks do demonstrate a high correlation with the temperature rhythm, peaking during the late afternoon and evening<sup>6,7</sup>. However, those tasks which are cognitively more complex, especially requiring verbal reasoning and/or short-term memory, tend to peak during the late morning or midday<sup>8</sup>. Moreover, differences have also been found for the rate of phase adjustment of various tasks to shiftwork and transmeridian flight (jet-lag). These earlier results thus suggest indirectly that circadian performance rhythms may be controlled by more than a single underlying oscillator<sup>9</sup>.

A multi-oscillator mathematical model<sup>4</sup> of the circadian system was used to predict the boundary at which desynchronization would occur. A 22-yr-old healthy right-handed man lived for 52 calendar days in a temporal isolation facility of the Laboratory of Human Chronophysiology; he was completely isolated from all normal time cues, but was not in social isolation<sup>10</sup>. After 9 days of his customary 24 h sleep/wake schedule, he was subjected to a single 5-h phase delay (of sleep onset time), followed immediately by 40 'days' on an exact 25.8-h sleep/wake and meals schedule. As predicted, this produced a desynchronization, with the subject's circadian temperature rhythm holding to a mean period of 24.8 h, in contrast to the imposed 25.8 h period of his sleep/wake cycle. Such a split is noteworthy in view of the comparatively small lengthening of the biological 'day', and (as will be discussed in a further publication) represents a strong validation of the model.

Rectal temperature was automatically recorded every minute of the study. Each performance test was administered on average five times per day (when the subject voided urine, ate solid food, and at the onset and end of each segment of wakefulness). Each performance test battery comprised: (1) a 32-trial

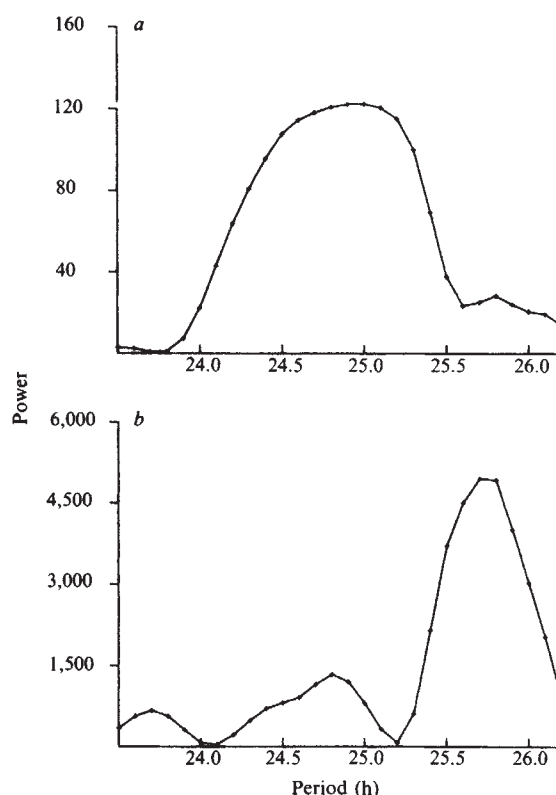


**Fig. 1** Power spectra of rectal temperature produced by periodogram (minimum variance) analysis of all 63,130 minute-by-minute temperature readings ( $\Delta$ ), and by 'bandpass filter' analysis of the 155 temperature values associated with the performance testing sessions (each being the mean of the five preceding minutes) (+). The power is expressed in squared units of  $0.01^\circ\text{F}$ .

search task; (2) 32 trials of a verbal reasoning task requiring the manipulation of grammatical transformations (modified from the Baddeley reasoning test)<sup>11,12</sup>; (3) a manual dexterity task (adapted from the Purdue pegboard test; Model 32020, Lafayette Instrument Co., Indiana) with the dominant hand; and (4) a repeat of the pegboard test with the non-dominant hand, in that order. All testing was carried out 'on-line' using a computer which presented material to the subject on a CRT terminal and timed responses to the nearest 10 ms. The subject's behaviour during testing was monitored by using closed-circuit TV. Here we describe the results from the pegboard test (dominant hand) and the verbal reasoning test.

There were 155 performance testing sessions. The measurements taken were the time taken to fill 25 holes of the pegboard with individual pegs, and the accumulated response latencies to the 32 trials of the verbal reasoning task. These times averaged  $36.4 \pm 2.9$  s ( $\pm$ s.d.) and  $54.8 \pm 12.6$  s, respectively. We determined the mean core temperature for the 5 min preceding each test session. A comparison was then made between a Fourier-based 'bandpass' filter analysis<sup>13</sup> of the 155 mean temperature values, and a more extensive periodogram ('minimum variance') analysis of all 63,130 minute-by-minute temperature readings<sup>14</sup>. Essentially identical power spectra resulted (Fig. 1), with peaks at 24.8- and  $\sim$ 25.8-h periods, demonstrating the validity of the bandpass filtering technique.

This bandpass filtering technique was then used to estimate the relative contribution or 'power' of the temperature oscillator (period 24.8 h), and the sleep/wake oscillator (period 25.8 h). The method involved obtaining least-squares-fitted sinusoids for periods between 23.5 h and 26.2 h in steps of 0.1 h. Each fit gave values of amplitude, phase, mean level, and two measures of statistical reliability—the probability of the rhythm having a zero amplitude ( $P_{ZA}$ ), and an  $F$  ratio of explained to unexplained variance<sup>15</sup>. Following the usual convention, the square of the amplitude was used as an index of the 'power' at that periodicity. The resultant power spectra (Fig. 2) demonstrated that a difference in the circadian behaviour emerged between the two tasks. The power spectrum of the manual dexterity task showed a dominant peak at  $\sim$ 24.8 h ( $P_{ZA} = 0.003$ ,  $F(2,152) = 7.20$ ,  $P < 0.001$ ), with very little power at a period of 25.8 h ( $P_{ZA} = 0.463$ ,  $F(2,152) = 0.86$ ,  $P > 0.25$ ). By contrast, the reasoning task had frequency peaks at both 24.8 h ( $P_{ZA} = 0.042$ ,  $F(2,152) = 3.24$ ,  $P < 0.05$ ) and 25.8 h ( $P_{ZA} < 0.0001$ ,  $F(2,152) = 16.45$ ,  $P < 0.001$ ), the 25.8 h peak being the greater. Thus, the manual dexterity task seems to depend only on the temperature oscillator, whereas the verbal reasoning task appears to depend on both sleep/wake and temperature oscillators. This conclusion was supported when the amplitudes of the (educated) time of day curves were compared for day lengths of 24.8 h and 25.8 h, and when the phase estimates from the



**Fig. 2** Power spectra of performance speed in the manual dexterity (pegboard) task (a) and in the verbal reasoning task (b). Each power spectrum was produced by a bandpass filtering technique applied to the 155 latencies. The power is expressed in squared units of 0.1 s.

bandpass analysis were considered. For the manual dexterity task the range (that is, maximum–minimum) of the 24.8-h educated rhythm was much larger than that of the 25.8-h educated rhythm (4.19 s versus 0.84 s), but for the reasoning task the two ranges were similar (10.57 s versus 13.57 s, respectively). The manual dexterity task showed best performance at about the same time (within a 24.8-h day) as the highest body temperature, whereas the verbal reasoning task showed best performance fairly soon after waking (based on a 25.8-h day).

In both jet-lag and shiftwork situations, the temperature rhythm takes considerably longer to phase-adjust than the sleep/wake cycle<sup>16</sup>. The association of the dexterity task with the temperature oscillator is thus in agreement with the finding that the circadian rhythm in manual dexterity performance takes longer to adjust to a phase shift than that in verbal reasoning performance<sup>17</sup>.

Contemporary research by Folkard, Wever *et al.* (personal communication) in Europe using the 'fractional desynchronization' technique has also demonstrated that different performance rhythms can run at different periods. A possible explanation for the present results may be that the performance strategy by which a manual dexterity task is approached is related to the temperature oscillator, perhaps through a vigilance–motivational mechanism, whereas the more complex verbal reasoning performance depends on those mechanisms as well as on changes in information processing capacity as the waking day progresses, that is, requiring a certain amount or type of sleep to 'reset' it.

In conclusion, these results indicate that it is as incorrect to speak of a single performance rhythm as it is to speak of a single physiological rhythm. Clearly, different types of task can exhibit different circadian performance rhythms, and these may be under the control of different underlying brain oscillators.

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- Conroy, R. T. W. L. & Mills, J. N. *Human Circadian Rhythms* (Churchill, London, 1970).
- Weitzman, E. D. & Czeisler, C. A. *Neurosecretion and Brain Peptides* (eds Martin, J. B., Reichlin, S. & Bick, K. L.) 475-499 (Raven, New York, 1981).
- Wever, R. *Int. J. Chronobiol.* **3**, 19-55 (1975).
- Kronauer, R. E., Czeisler, C. A., Pilato, S. F., Moore-Ede, M. C. & Weitzman, E. D. *Am. J. Physiol.* **242**, R3-R17 (1982).
- Kleitman, N. & Jackson, D. P. *J. appl. Physiol.* **3**, 309-328 (1950).
- Colquhoun, W. P. *Handbook of Behavioral Neurobiology* (ed. Aschoff, J.) 333-348 (Plenum, New York, 1981).
- Blake, M. J. F. *Psychon. Sci.* **9**, 349-350 (1967).
- Folkard, S. & Monk, T. H. *Br. J. Psychol.* **71**, 295-307 (1980).
- Folkard, S. & Monk, T. H. *Psychophysiology* 1980 (eds Sinz, R. & Rosenzweig, M. R.) 541-548 (Elsevier Biomedical, North Holland, 1982).
- Weitzman, E. D., Czeisler, C. A., Zimmerman, J. C., Ronda, J. M. & Knauer, R. S. *Sleeping and Waking Disorders* (ed. Guilleminault, C.) 297-329 (Addison-Wesley, Menlo Park, 1982).
- Baddeley, A. D. *Psychon. Sci.* **10**, 341-342 (1968).
- Folkard, S. *Br. J. Psychol.* **66**, 1-8 (1975).
- Monk, T. H. & Fort, A. *Int. J. Chronobiol.* **8**, 193-222 (1983).
- Fookson, J., Weitzman, E. D., Czeisler, C. A., Zimmerman, J. C. & Ronda, J. *Proc. XV Int. Conf. of Int. Soc. for Chronobiol.* (Karger, New York, 1981).
- Monk, T. H. *Biological Rhythms, Sleep and Performance* (ed. Webb, W. B.) 27-57 (Wiley, Chichester, 1982).
- Aschoff, J., Hoffman, K., Pohl, H. & Wever, R. *Chronobiologia* **2**, 23-78 (1975).
- Hughes, D. G. & Folkard, S. *Nature* **264**, 432-434 (1976).

## Growth hormone stimulates the proliferation of cultured chondrocytes from rabbit ear and rat rib growth cartilage

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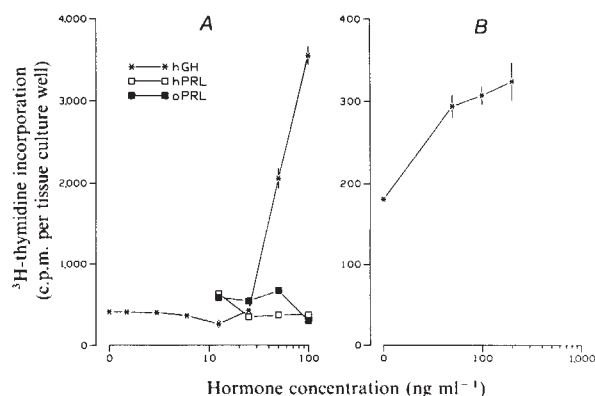
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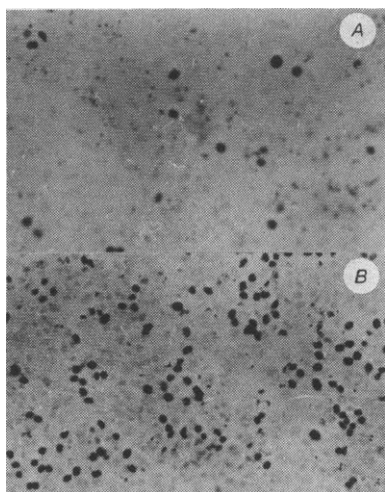
The effect of growth hormone (GH) on various growth processes is generally considered to be indirect, mediated by GH-dependent plasma factors<sup>1</sup>—somatomedins—which are produced mainly in the liver<sup>2-4</sup>. *In vitro*, somatomedins stimulate a number of processes<sup>5-10</sup> that apparently are associated with cell growth<sup>1</sup>. It has been difficult, however, to induce skeletal growth by the administration of somatomedins *in vivo*. Daily injections of a partially purified somatomedin preparation failed to induce accumulated longitudinal bone growth using the intravital marker tetracycline or by measuring the nose-to-tail length<sup>11,12</sup>. Administration of insulin-like growth factor I (IGF I) which is probably identical to somatomedin C<sup>13</sup>, to hypophysectomized rats has been reported to increase the width of the epiphyseal plate<sup>14</sup>. But although this suggests an *in vivo* effect of IGF I on longitudinal bone growth, such an effect has not been directly demonstrated. Recently, we reported that local administration of human GH (hGH) into the proximal cartilage growth plate of the tibia of hypophysectomized rats stimulated longitudinal bone growth on the side injected with the hormone<sup>15</sup>. Furthermore, we have identified specific binding sites for hGH in cultured chondrocytes from rabbit ear and epiphyses<sup>16</sup>. Here, we show that hGH, but not the structurally related polypeptides ovine prolactin or human prolactin, stimulates DNA synthesis in chondrocytes from rabbit ear and from rat rib growth plate, cultured in a chemically defined medium without the addition of serum. Our results suggest that GH directly initiates proliferation in mammalian chondrocytes.

Ear chondrocytes were isolated as described previously<sup>16,17</sup> from 4-week-old male New Zealand White rabbits. More than

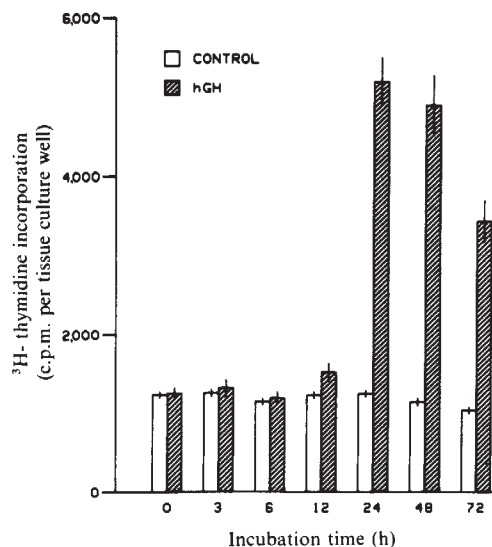


**Fig. 1** Effect of hGH, oPRL and hPRL on thymidine incorporation in chondrocytes from rabbit ear (A) and the effect of hGH on thymidine incorporation in chondrocytes from rat rib growth plate (B). After collagenase digestion, the cells were plated in 96-well tissue culture plates in F-12 containing 10% NCS (0.2 ml per well) and were allowed to attach overnight. The medium was then changed to MCDB-104 and the cells were preincubated for 24 h. The cells were then incubated in fresh MCDB-104 for 24 h in the absence and presence of various concentrations of hGH, oPRL and hPRL. After another 24 h of incubation, the medium was changed to fresh MCDB-104 with the same addition plus <sup>3</sup>H-thymidine (5 µCi ml<sup>-1</sup>; specific activity 9.9 Ci mol<sup>-1</sup>). The incubation was terminated by three washes of the cells in phosphate-buffered saline (PBS) before treatment with 0.5% Triton X-100 (100 µl per well). The chondrocytes were collected in a multiple cell harvester (Skatron) on glass-fibre filters. The filters were dried at 70°C and then counted in 2.5 ml of Lipoluma: Lumasolve: water, 10:1:0.2 (Lumac) in a Packard liquid scintillation spectrometer. Each point is the mean of six determinations ± s.e.m. Data are shown from an experiment using ear chondrocytes from one rabbit (A), or chondrocytes from the pooled rib growth cartilage of 10 rats (B). The experiment presented in A was performed three times, and the experiment presented in B twice, with similar results.

90% of the cells obtained were chondrocytes showing different degrees of hypertrophy and less than 10% were small cells from the peripheral part of the cartilage, presumably chondroblasts. These latter cells are not fibroblasts since they produce cartilage-like proteoglycans and do not synthesize large amounts of dermatan sulphate or type I collagen<sup>18</sup>. Chondrocytes from rat rib growth plates were isolated from 5-week-old male rats of the Sprague-Dawley strain (Anticimex) as described by Shimomura *et al.*<sup>19</sup>. In brief, ear cartilages carefully dissected under a stereo microscope clean of skin, muscle and perichondrium, or whole pieces of rat rib growth plate were digested overnight at 37°C in 0.1% (w/v) *Clostridium* collagenase in complete medium (see below). After approximately 24 h incubation, the cells were washed twice with serum-free medium, counted in a haemocytometer, and plated in 24-well (Falcon) or 96-well (Nunc) tissue culture plates. The cells were plated at a density of about  $3 \times 10^5$  cells per cm<sup>2</sup> in F-12 medium (Gibco Bio-Cult) supplemented with 10% (v/v) heat inactivated newborn calf serum (NCS; Flow Laboratories). The medium was further supplemented with HEPES (10 mM), trimethylamino ethane sulphonic acid (10 mM), gentamycin (100 µg ml<sup>-1</sup>) and L-ascorbic acid (50 µg ml<sup>-1</sup>) (all substances from Sigma). The cells were cultured at 37°C in an atmosphere of air containing 5% CO<sub>2</sub>. On the day after plating the medium was changed to serum-free MCDB-104 (Gibco Bio-Cult) and the cells were then preincubated for 24-48 h to establish growth arrest. After preincubation, the medium was changed to fresh MCDB-104 with the addition of hormones at various concentrations in the experimental groups, the cultures then being incubated for various times. At either 6 or 24 h before the termination of incubation, [<sup>3</sup>H]thymidine (24 Ci mmol<sup>-1</sup>, Amersham) was added to the cultures and the incorporation of <sup>3</sup>H-thymidine was determined by liquid scintillation counting or by autoradiography. Highly purified hGH (2.0 IU mg<sup>-1</sup>) was



**Fig. 2** Autoradiographs of  $^3\text{H}$ -thymidine-labelled chondrocytes from rabbit ear cultured in the absence and presence of hGH. The chondrocytes were plated in 24-well tissue culture plates in F-12 containing 10% NCS (1 ml per well) and allowed to attach overnight. The medium was then changed to MCDB-104 and the cultures were preincubated for 24 h. The cells were then incubated in fresh MCDB-104 for 48 h in absence and presence of hGH. During the last 24 h of the incubation the cells were exposed to  $^3\text{H}$ -thymidine ( $2 \mu\text{Ci ml}^{-1}$ ; specific activity  $5.2 \text{ Ci mmol}^{-1}$ ). At the end of the incubation the cultures were triple rinsed with PBS, fixed overnight at  $4^\circ\text{C}$  with 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) containing 0.05 M sucrose, and, finally, rinsed again in the same buffer without glutaraldehyde. Staining was done with 2% orcein in 70% acetic acid for 2 h. The cultures were then rinsed with ethanol and dried. The bottoms of the wells were punched out and mounted on microscopic slides. Autoradiography was performed with the dip slide method, using Kodak NTB2 nuclear track emulsion. The specimens were exposed at  $4^\circ\text{C}$  for 3 days and developed in Kodak D-19. **A**, Control culture. **B**, Culture treated with  $100 \text{ ng ml}^{-1}$  hGH. The experiment was performed three times with similar results using different rabbits.



**Fig. 3** Time course for the stimulatory effect of hGH on  $^3\text{H}$ -thymidine incorporation in chondrocytes from rabbit ear. The cells were plated in 96-well tissue culture plates in F-12 containing 10% NCS (0.2 ml per well) and allowed to attach overnight. They were then preincubated for 48 h in MCDB-104. The cultures were then incubated for various periods in the absence or presence of hGH ( $100 \text{ ng ml}^{-1}$ ). For cultures incubated for 48 or 72 h, the medium was changed every 24 h. During the last 6 h of the incubation, the cells were pulse-labelled with  $^3\text{H}$ -thymidine ( $8 \mu\text{Ci ml}^{-1}$ ; specific activity  $12.8 \text{ Ci mmol}^{-1}$ ). The cultures were collected and counted as described in the legend to Fig. 1. Each point is the mean of 24 cultures  $\pm$  s.e.m. Data from an experiment using chondrocytes from one rabbit are shown. The experiment was repeated twice using different rabbits, and resulted in similar findings.

prepared according to Roos *et al.*<sup>20</sup>. Human prolactin (hPRL) was a gift from Kabi AB and ovine prolactin (oPRL, Lot NIH-P-S-3) was obtained from the NIADDK (Bethesda, Maryland).

Chondrocytes from rabbit ear were cultured with various concentrations of hGH, oPRL and hPRL for 48 h and labelled with  $^3\text{H}$ -thymidine during the last 24 h of culture. As shown in Fig. 1A, hGH caused a dose-dependent increase in thymidine incorporation, whereas oPRL and hPRL exerted no effect. The minimal effective concentration of hGH was  $50 \text{ ng ml}^{-1}$ . Figure 1B shows that hGH in a similar experiment stimulated the thymidine incorporation in chondrocytes from rat rib growth plate at all concentrations of hGH tested. A concentration of hGH of  $50 \text{ ng ml}^{-1}$  evoked an apparent maximal effect in these cells. Figure 2 shows autoradiographs of cultured chondrocytes from rabbit ear that were incubated with and without hGH ( $100 \text{ ng ml}^{-1}$ ) for 48 h;  $^3\text{H}$ -thymidine was present during the last 24 h. As can be seen in this figure, hGH had a marked stimulatory effect. To study the time course for this GH effect on thymidine incorporation, cultured chondrocytes were pulse-labelled with  $^3\text{H}$ -thymidine for 6 h at various times after the start of the incubation. Figure 3 shows that the stimulatory effect of hGH became apparent between 12 and 24 h after the start of culture. The effect persisted for 72 h with an apparent decrease between 48 and 72 h.

The results from the present study demonstrate that hGH, but not hPRL or oPRL, stimulates DNA synthesis in cultured chondrocytes from rabbit ear and rat rib growth plate, when a chemically defined culture medium without serum is used. A stimulatory effect was apparent with a hGH concentration of  $50 \text{ ng ml}^{-1}$ . This concentration frequently can be recorded in the plasma during episodes of GH secretion both in the rabbit

and the rat<sup>21,22</sup> and is therefore well within the physiological range.

Several other investigators have exposed isolated chondrocytes to GH without being able to show any stimulatory effect on cell proliferation<sup>23-25</sup>. The apparent discrepancy compared with our results could be due to many different causes. In the present study, the isolated cells were allowed to attach to plastic tissue culture plates and were preincubated for 24–48 h before GH exposure. Other investigators<sup>23-25</sup> have used freshly isolated chondrocytes in suspension. Hypothetically the isolation procedure *per se* could activate different mitogenic processes that could mask other mitogenic effects. One of the cited studies<sup>25</sup> used embryonic human cartilage as a source of chondrocytes. Since GH is not required for normal growth during fetal life<sup>26</sup>, it is possible that the cells used had not developed a responsiveness to the hormone. Another factor that might influence the ability of GH to stimulate cultured chondrocytes is the composition of the tissue culture medium. The fact that the chondrocytes in the present study were cultured in a medium rich in cofactors and nutrients (MCDB-104) might be important in this regard.

Although most investigators studying the effects of GH on pieces of cartilage tissue have been unable to find any effects<sup>2</sup>, there are some earlier reports that GH, in certain conditions, exerts both stimulatory and inhibitory effects. Almqvist<sup>27</sup> found that hGH stimulated radioactive sulphate incorporation into rat costal cartilage when serum from normal or hypophysectomized humans was present in the incubation medium. In contrast, Smith *et al.*<sup>28</sup> reported that GH depressed glycosaminoglycan synthesis in pieces of bovine articular cartilage cultured for 1 or 3 days.

The present finding, that hGH stimulates DNA synthesis when the chondrocytes are cultured in a chemically defined medium without addition of serum, shows that circulating plasma growth factors, such as somatomedins, are not necessarily required for the stimulatory effect of hGH.



However, it is possible that the cultured chondrocytes themselves, in response to GH *in vitro*, produce somatomedins or other growth factors that may be of importance for the effect of the hormone. Interestingly, it has been shown that the stimulatory effect of hGH *in vitro* on the growth of cultured human fibroblasts is not expressed when the culture medium is changed frequently<sup>29</sup>. This suggests that fibroblasts indeed secrete 'factors' into the incubation medium that are important for the effect of GH on fibroblasts. The extent to which the effect of GH on chondrocyte growth is mediated or modulated

by 'local somatomedins' remains to be shown. Nevertheless, our results demonstrate that GH has the ability to interact directly with chondrocytes from rabbit ear and rat rib growth cartilage, causing an increased DNA synthesis.

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1. Salmon, W. D. & Daughaday, W. H. *J. Lab. clin. Med.* **49**, 825-836 (1957).
2. Phillips, L. S. & Vassilopoulou-Sellin, R. *New Engl. J. Med.* **302**, 371-380 (1980).
3. Daughaday, W. H. in *Endocrine Control of Growth* (ed. Daughaday, W. H.) 1-24 (Elsevier, Amsterdam, 1981).
4. Daughaday, W. H. *et al. Nature* **235**, 107 (1972).
5. Kostyo, J. L., Hotchkiss, J. & Knobil, E. *Science* **130**, 1653-1654 (1959).
6. Kostyo, J. L. & Knobil, E. *Endocrinology* **65**, 395-401 (1959).
7. Ahrén, K. & Hjalmarson, Å. in *Growth Hormone* (eds Pecile, A. & Müller, E.) 143-152 (Excerpta Medica, Amsterdam, 1968).
8. Albertsson-Wikland, K. & Isaksson, O. *Metabolism* **25**, 747-759 (1976).
9. Jefferson, L. S., Schworer, C. M. & Tolman, E. L. *J. biol. Chem.* **250**, 197-204 (1975).
10. Hjalmarson, Å., Isaksson, O. & Ahrén, K. *Am. J. Physiol.* **217**, 1795-1802 (1969).
11. Fryklund, L., Uthne, K. & Sievertsson, H. *Biochem. biophys. Res. Commun.* **61**, 957-962 (1974).
12. Thorngren, K.-G., Hansson, L. I., Fryklund, L. & Sievertsson, H. *Molec. cell. Endocr.* **6**, 217-221 (1977).
13. Hintz, R. L., Liu, F. & Rinderknecht, E. *J. clin. endocr. Metab.* **51**, 672-673 (1980).

14. Schoenle, E., Zapf, J., Humbel, R. E. & Froesch, E. R. *Nature* **296**, 252-253 (1982).
15. Isaksson, O. G. P., Jansson, J. O. & Gause, I. A. M. *Science* **216**, 1237-1239 (1982).
16. Edén, S., Isaksson, O. G. P., Madsen, K. & Friberg, U. *Endocrinology* **112**, 1127-1129 (1983).
17. Madsen, K. & Lohmander, S. *Archs Biochem. Biophys.* **196**, 192-198 (1979).
18. Madsen, K., Moskalewski, S., von der Mark, K. & Friberg, U. *Dev. Biol.* **96**, 63-73 (1983).
19. Shimomura, Y., Yoneda, T. & Suzuki, F. *Calcified Tissue Res.* **19**, 179-187 (1975).
20. Roos, P., Fevold, H. R. & Gemzell, C. A. *Biochim. biophys. Acta* **74**, 525-531 (1963).
21. McIntyre, H. B. & Odell, W. D. *Neuroendocrinology* **16**, 8-21 (1974).
22. Edén, S. *Endocrinology* **105**, 555-560 (1979).
23. Ash, P. & Francis, M. J. O. *J. Endocr.* **66**, 71-78 (1975).
24. Ashton, I. K. & Francis, M. J. O. *J. Endocr.* **74**, 205-212 (1977).
25. Ashton, I. K. & Francis, M. J. O. *J. Endocr.* **76**, 473-477 (1978).
26. Cheek, D. B. & Hill, D. E. in *Handbook of Physiology*, Vol. IV, Pt 2 (eds Knobil E. & Sawyer W. H.) 159-185 (American Physiology Society, Washington, 1974).
27. Almquist, S. *Acta endocr.* **36**, 31-50 (1961).
28. Smith, T. W. D., Duckworth, T., Bergenholz, A. & Lempberg, R. K. *Nature* **253**, 269-271 (1975).
29. Clemmons, D. R. & Van Wyk, J. J. *J. cell. Physiol.* **106**, 361-367 (1981).

## Human preprovasoactive intestinal polypeptide contains a novel PHI-27-like peptide, PHM-27

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Vasoactive intestinal polypeptide (VIP), a 28-amino acid peptide originally isolated from porcine duodenum<sup>1</sup>, is present not only in gastrointestinal tissues but also in neural tissues, possibly as a neurotransmitter, and exhibits a wide range of biological actions (for example, relaxation of smooth muscle, stimulation of intestinal water and electrolyte secretion and release of insulin, glucagon and several anterior pituitary hormones)<sup>2-5</sup>. As the structure of porcine and bovine VIP shows several similarities to those of mammalian glucagon, secretin and gastric inhibitory peptide (GIP), VIP is considered to be a member of the glucagon-secretin family<sup>2</sup>. Recently, we have found that VIP is synthesized from a precursor, pro-VIP (molecular weight ( $M_r$ ) 17,500), in human neuroblastoma cells and that the primary translation product of the mRNA encoding VIP is prepro-VIP ( $M_r$  20,000)<sup>6</sup>. In an attempt to elucidate the primary structure of the precursor, we have now cloned the DNA sequence complementary to the mRNA coding for human VIP and analysed the nucleotide sequence. The entire amino acid sequence of the precursor, deduced from the nucleotide sequence, indicates that the precursor protein contains not only VIP but also a novel peptide of 27 amino acids. The peptide, designated PHM-27, differs by only 2 amino acids from PHI-27, a peptide recently isolated from porcine intestine<sup>7</sup>, and is also closely related in sequence to VIP.

Poly(A)-containing RNA was isolated from human neuroblastoma cells as described previously<sup>6</sup>. As the content of mRNA for the VIP precursor in the poly(A)-containing RNA was found to be only 0.12% by the cell-free translation assay using a rabbit reticulocyte lysate followed by immunoprecipitation with anti-VIP antiserum, the poly(A)-containing RNA was fractionated by sucrose gradient centrifugation<sup>8,9</sup>. Double-

stranded cDNA was synthesized using the partially purified mRNA as a template and inserted into the *Pst*I endonuclease cleavage site of the plasmid pBR322 by the oligo(dG)-oligo(dC) tailing method. *Escherichia coli*  $\chi$ 1776 was transformed with the recombinant DNA. To identify the transformants containing the recombinant plasmid carrying the cDNA sequence for the VIP precursor, the plasmid DNAs were isolated from the transformants and analysed by a hybridization-translation assay. A plasmid DNA positive in the assay was designated as pVIP-1, and the cDNA in pVIP-1 was subjected to nucleotide sequence analysis. As the cDNA insert (nucleotide residues 53-1,279 in Fig. 1) did not contain the entire protein-coding sequence, we determined the sequence of the 5'-terminal region by the primer extension method. The 5'-<sup>32</sup>P-labelled anticoding strand of the *Hgi*AI-*Hae*III fragment (nucleotide residues 53-78 in Fig. 1) that hybridized with the poly(A)-containing RNA from human neuroblastoma cells, was elongated by reverse transcriptase. The nucleotide sequence of the elongated DNA was also analysed.

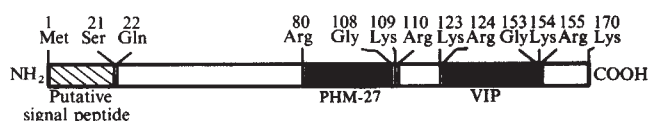
Figure 1 shows the nucleotide sequence of the mRNA for the precursor protein. Nucleotide residues 53-1,279 were deduced from the nucleotide sequence of the cloned cDNA and nucleotides -30-52 were deduced from the nucleotide sequence of the elongated cDNA with the primer. The amino acid sequence (residues 125-152) deduced from the nucleotide residues 373-456 corresponds precisely to the amino acid sequence of porcine and bovine VIP<sup>2</sup>. Although the amino acid sequence of human VIP has not been determined, the amino acid composition of human VIP isolated from colon tissue is identical to those of porcine and bovine VIP<sup>10</sup>. Therefore, it is reasonable to assume that the amino acid sequence (residues 125-152) corresponds to that of human VIP. The amino-terminus of human VIP is preceded by a pair of basic amino acid residues (Lys-Arg) known to be frequent sites for the post-translational processing of hormone precursors<sup>11</sup>. A glycine residue and a pair of basic amino acid residues lie adjacent to the carboxy-terminal Asn residue. As with other polypeptide precursors<sup>12-14</sup>, the glycine residue seems to serve as an amino donor to the carboxy-terminus of human VIP, because both porcine and bovine VIP contain a carboxy-terminal Asn-NH<sub>2</sub> residue<sup>2</sup>. The identity of porcine, bovine and human VIP suggests that VIP, like glucagon, may have been conserved during evolution in mammals.

The most interesting finding is that the deduced amino acid sequence at residues 81-107, which shows a remarkable similarity to that of VIP, is almost identical to that of porcine PHI-27

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**Fig. 1** Primary structure of human prepro-VIP/PHM-27 mRNA and protein. Nucleotide residues are numbered in the 5' to 3' direction, beginning with the first residue of the AUG triplet encoding the initiator methionine, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The deduced amino acid sequence is given above the nucleotide sequence, and amino acid residues are numbered beginning with the initiator methionine. The VIP and PHM-27 sequences are boxed. The two translation termination codons, UGA (nucleotides 511–516), were found at a site 55 nucleotides downstream from the carboxy-terminus of VIP. The translational initiation site was assigned to the methionine codon, AUG (nucleotides 1–3), that is, the first AUG triplet in the deduced mRNA sequence, because the sequence of ~20 amino acid residues starting with this methionine (residue 1) exhibits a feature characteristic of the signal peptide at the amino-terminal region of a secretory protein<sup>11</sup>, and the molecular weight (19,169) of the precursor protein (170 amino acid residues) calculated by this assignment was consistent with that of the precursor protein, prepro-VIP ( $M_r$  20,000), estimated by SDS-polyacrylamide gel electrophoresis<sup>6</sup>. It has been shown that the signal peptide generally contains a region rich in hydrophobic amino acid residues with large side chains and terminates in a residue having a small neutral side chain (alanine, serine or glycine)<sup>11</sup>. Therefore, a possible site for cleavage of the signal peptide in the precursor seems to exist after the serine residue at position 21 (or after the serine or alanine residue at positions 24 or 25). A possible glycosylation site, Asn-X-Thr<sup>20</sup>, is located at amino acid residues 68–70, suggesting that the VIP/PHM-27 precursor protein may be a glycoprotein. The 3'-noncoding region of the mRNA is unusually long (>700 nucleotides excluding the poly(A) tract) and contains three copies of the sequence AAUAAA (nucleotide residues 644–649, 978–983 and 1,260–1,265) which precede the site of polyadenylation in many eukaryotic mRNAs<sup>21</sup>.

**Methods:** Poly(A)-containing RNA, which was isolated from human neuroblastoma cells as described previously<sup>6</sup>, was applied to a linear 15–30% sucrose gradient, and centrifuged at 60,000 r.p.m. at 20 °C for 6 h in a Beckman SW 60 Ti rotor<sup>8,9</sup>. The 12–13S fraction which contained the mRNA for the VIP precursor was ethanol-precipitated. The content of the mRNA for the precursor in the partially purified mRNA was found to be ~1.5%. Single-stranded cDNA was synthesized from the partially purified mRNA with avian myeloblastosis virus reverse transcriptase<sup>22</sup> (provided by Dr Y. Ikawa, Cancer Institute, Tokyo). Double-stranded cDNA was synthesized from single-stranded cDNA with a large fragment of *E. coli* DNA polymerase I (BRL)<sup>22</sup>. The double-stranded cDNA was treated with  $S_1$  nuclease<sup>23</sup> (Sankyo) and dC-tailed with calf thymus terminal transferase<sup>24</sup> (P-L Biochemicals). The dC-tailed double-stranded cDNA was then annealed to dG-tailed *Pst*I-cut pBR322<sup>24</sup>. Transformation of *E. coli*  $\chi$ 1776 was carried out with the recombinant DNA<sup>25</sup>. About 1,500 tetracycline-resistant transformants were obtained and then screened by the colony hybridization method<sup>26</sup> with <sup>32</sup>P-labelled DNA complementary to the partially purified mRNA, yielding about 200 hybridization-positive clones. The plasmid DNA (about 2  $\mu$ g) prepared from the hybridization-positive clone was immobilized to a dibenzylloxymethyl (DBM) paper<sup>27</sup> (1 cm<sup>2</sup>). 3  $\mu$ g of the poly(A)-containing RNA from human neuroblastoma cells with 30  $\mu$ g of rat liver RNA and 10  $\mu$ g of polyadenylic acid (P-L Biochemicals) were hybridized with the plasmid DNA immobilized on a DBM paper in 15  $\mu$ l of hybridization buffer<sup>27</sup> (50% formamide, 0.6 M NaCl, 4 mM EDTA, 40 mM Tris-HCl, pH 7.8, 0.1% SDS) at 37 °C for 18 h. After hybridization, the paper was washed three times with 3 ml of the hybridization buffer at 37 °C for 1 h and washed once with 3 ml of 0.3 M NaCl containing 2 mM EDTA and 20 mM Tris-HCl, pH 7.8, at 25 °C for 30 min. The hybridized RNA was eluted from the washed paper with an elution buffer<sup>27</sup> (99% formamide, 10 mM Tris-HCl, pH 7.8) at 65 °C for 5 min and precipitated with 10  $\mu$ g of wheat germ tRNA (BRL) using ethanol. The precipitate was washed twice with 1 ml of 70% ethanol, dissolved in 10  $\mu$ l of H<sub>2</sub>O and translated by the cell-free translation system of a rabbit reticulocyte lysate<sup>6</sup> with <sup>35</sup>S-methionine (1,500 Ci mmol<sup>-1</sup>; Amersham). The translation product was immunoprecipitated with antiserum to synthetic porcine VIP<sup>28</sup> and analysed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography<sup>6</sup>. A plasmid DNA positive in the hybridization-translation assay was designated as pVIP-1. The cDNA insert in pVIP-1 was fragmented by restriction endonucleases. DNA sequence analysis on both strands was performed by the method of Maxam and Gilbert<sup>29</sup>. For synthesis of DNA complementary to the 5'-terminal region of the mRNA for the VIP precursor, the *Hgi*AI-*Hae*III fragment (nucleotide residues 53–78) of the cDNA insert was denatured and applied to an 8% polyacrylamide sequence gel in the presence of 8.3 M urea<sup>29</sup> for strand separation. The 5' end of the 26-nucleotide long anticoding strand of the fragment was dephosphorylated with alkaline phosphatase (P-L Biochemicals) and labelled with T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan) and 5'-[ $\gamma$ -<sup>32</sup>P]ATP (3,000 Ci mmol<sup>-1</sup>; Amersham)<sup>29</sup>. The primer extension reaction was carried out according to the method of Nagata *et al.*<sup>30</sup>. The <sup>32</sup>P-labelled anticoding strand (6  $\times$  10<sup>6</sup> c.p.m.) was mixed with 75  $\mu$ g of the poly(A)-containing RNA from neuroblastoma cells in 20  $\mu$ l of 6 mM NaCl, 6 mM HEPES-KOH, pH 7.5, 0.6 mM EDTA. After incubation at 100 °C for 90 s, 5  $\mu$ l of 0.84 M NaCl were added and the mixture was incubated at 50 °C for 2 h. After incubation, 75  $\mu$ l of the solution (53 mM Tris-HCl, pH 7.5, 6.6 mM dithiothreitol, 6.5 mM MgCl<sub>2</sub>, 0.64 mM dGTP, dATP, TTP and dCTP) containing 200 units of human placenta ribonuclease inhibitor (Wako Pure Chemicals, Osaka, Japan) and 20 units of reverse transcriptase (Seikagaku Kogyo Co., Tokyo) were added, and the incubation was carried out at 37 °C for 90 min. The mixture was treated with phenol and ethanol-precipitated. The precipitate was subjected to an 8% polyacrylamide sequence gel electrophoresis in the presence of 8.3 M urea<sup>29</sup>. The elongated DNA (corresponding to ~250 nucleotides length) was eluted from the gel and sequenced following the method of Maxam and Gilbert<sup>29</sup>.



**Fig. 2** Schematic representation of the structure of human prepro-VIP/PHM-27. Numbering of the amino acid residues is as in Fig. 1. The putative signal peptide (1–21) is presented by the cross-hatched bar. VIP (125–152) and PHM-27 (81–107) are represented by the closed bars. The amino acid residues at the post-translational processing site are shown.



**Fig. 3** Comparison of the amino acid sequences of VIP, PHM-27, PHI-27 and hpGRF. The amino acid sequences of human VIP and PHM-27 were deduced from the nucleotide sequence of human prepro-VIP/PHM-27 mRNA described in Fig. 2.

|                                 |                                                                                                          |    |    |    |    |
|---------------------------------|----------------------------------------------------------------------------------------------------------|----|----|----|----|
| VIP (porcine,<br>bovine, human) | 1                                                                                                        | 10 | 20 | 28 |    |
|                                 | HisSerAspAlaValPheThrAspAsnTyrThrArgLeuArgLysGlnMetAlaValLysTyrLeuAsnSerIleLeuAsnNH <sub>2</sub>         |    |    |    |    |
| PHM-27                          | 1                                                                                                        | 10 | 20 | 27 |    |
|                                 | HisAlaAspGlyValPheThrSerAspPheSerLysLeuLeuGlyGlnLeuSerAlaLysTyrLeuGluSerLeuMetNH <sub>2</sub>            |    |    |    |    |
| PHI-27                          | 1                                                                                                        | 10 | 20 | 27 |    |
|                                 | HisAlaAspGlyValPheThrSerAspPheSerArgLeuLeuGlyGlnLeuSerAlaLysTyrLeuGluSerLeuIleNH <sub>2</sub>            |    |    |    |    |
| hpGRF                           | 1                                                                                                        | 10 | 20 | 27 | 44 |
|                                 | TyrAlaAspAlaIlePheThrAsnSerTyrArgLysValLeuGlyGlnLeuSerAlaArgLysLeuLeuGlnAspIleMet-----LeuNH <sub>2</sub> |    |    |    |    |

(a peptide (P) having amino-terminal histidine (H), carboxy-terminal isoleucine (I) amide and 27 amino acid residues), a member of the glucagon–secretin family recently isolated from porcine upper intestinal tissue<sup>7</sup>. The amino acid sequence differs from that of porcine PHI-27 only at residues 92 (Arg→Lys) and 107 (Ile→Met). As both these amino acid substitutions can be explained by one-point mutation of the codons, it is plausible to assume that the amino acid sequence at residues 81–107 corresponds to that of human PHI-27. However, we cannot exclude the possibility that authentic PHI-27 may also be found in man and coded for by a different gene from that encoding the VIP precursor. Although it has been shown that a pair of basic amino acid residues are frequent sites for the post-translational processing of hormone precursors<sup>11</sup>, the amino-terminus of the peptide is preceded by one basic amino acid residue, Arg, at position 80. A similar example is reported in the cleavage between neurophysin II and the glycopeptide from the precursor protein<sup>14</sup>. The carboxy-terminal methionine residue is followed by a glycine residue and a pair of basic amino acid residues (Lys–Arg), just as with VIP. The glycine residue appears to serve as an amino donor to the carboxy-terminus of the peptide. Therefore, human PHI-27 is thought to be a 27-amino acid peptide (P) having an amino-terminal histidine (H) and a carboxy-terminal methionine (M) amide—PHM-27.

The present results indicate that VIP and PHM-27, two members of the glucagon–secretin family, are synthesized from the common precursor protein, prepro-VIP/PHM-27. Figure 2 shows the schematic representation of the structure of human prepro-VIP/PHM-27. The recent finding that immunoreactive VIP and immunoreactive PHI-27 occur in roughly equimolar concentrations in extracts from a variety of tissues from several species and that VIP-producing tumours also produce PHI-27, have suggested that VIP and PHI-27 might be co-synthesized in the same precursor<sup>15</sup>. On the other hand, immuno-histochemical studies have also revealed that there are some differences in the distribution of VIP- and PHI-27-immunoreactive cells in the rat hypothalamus, indicating that VIP and PHI-27 may not coexist in the same neurones<sup>16,17</sup>. Therefore, in the hypothalamic cells the synthesis of VIP and PHI-27 may be regulated at the post-translational processing step. Although VIP is preceded by a pair of basic amino acids (Lys–Arg) in the precursor protein, PHM-27 is preceded by only one basic amino acid (Arg). This difference may be important in the regulation of post-translational processing. Furthermore, both VIP and PHM-27 contain a pair of internal basic amino acid residues (Lys–Lys and Arg–Lys). These could reflect a differential tissue-specific post-translational processing pathway, yielding some 'cryptic' peptides.

Recently, human pancreatic growth hormone-releasing factor (hpGRF, 40 or 44 amino acid residues) has been isolated from a human pancreatic tumour that had caused acromegaly<sup>18,19</sup>. The amino-terminal region of hpGRF has been reported to have a remarkable sequence homology to porcine PHI-27<sup>18,19</sup>. PHM-27 deduced here has two additional residues (positions 12 and 27 in Fig. 3) common to the amino-terminal region of hpGRF.

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- Mutt, V. & Said, S. I. *Eur. J. Biochem.* **42**, 581–589 (1974).
- Said, S. I. (ed.) *Vasoactive Intestinal Peptide* (Raven, New York, 1982).
- Kato, Y. *et al. Endocrinology* **103**, 554–558 (1978).

- Vijayan, E., Samson, W. K., Said, S. I. & McCann, S. M. *Endocrinology* **104**, 53–57 (1979).
- Ottesen, B. *et al. Lancet* **ii**, 696 (1981).
- Obata, K. *et al. FEBS Lett.* **136**, 123–126 (1981).
- Tatemoto, K. & Mutt, V. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6603–6607 (1981).
- Itoh, N., Nose, K. & Okamoto, H. *Eur. J. Biochem.* **97**, 1–9 (1979).
- Okamoto, H. *Molec. cell. Biochem.* **37**, 43–61 (1981).
- Carlquist, M. *et al. Horm. metab. Res.* **14**, 28–29 (1982).
- Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J. & Tager, H. S. *Ann. N.Y. Acad. Sci.* **343**, 1–16 (1980).
- Jacobs, J. W., Goodman, R. H., Chin, W. W., Dee, P. C. & Habener, J. F. *Science* **213**, 457–459 (1981).
- Yoo, O. J., Powell, C. T. & Agarwal, K. L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1049–1053 (1982).
- Land, H., Schütz, G., Schmale, H. & Richter, D. *Nature* **295**, 299–303 (1982).
- Christofides, N. D. *et al. Lancet* **ii**, 1398 (1982).
- Hökfelt, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 895–898 (1983).
- Hökfelt, T., Fahrenkrug, J., Tatemoto, K., Mutt, V. & Werner, S. *Acta physiol. scand.* **116**, 469–471 (1982).
- Guillemin, R. *et al. Science* **218**, 585–587 (1982).
- Rivier, J., Spiess, J., Thorner, M. & Vale, W. *Nature* **300**, 276–278 (1982).
- Pless, D. D. & Lennarz, W. J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 134–138 (1977).
- Proudfoot, N. J. & Brownlee, G. G. *Nature* **263**, 211–214 (1976).
- Wickens, M. P., Buell, G. N. & Schimke, R. T. *J. biol. Chem.* **253**, 2483–2495 (1978).
- Wahli, W. *et al. Devl Biol.* **67**, 371–383 (1978).
- Gordon, J. I., Burns, A. T. H., Christmann, J. L. & Deeley, R. G. *J. biol. Chem.* **253**, 8629–8639 (1978).
- Norgard, M. V., Keem, K. & Monahan, J. J. *Gene* **3**, 279–292 (1978).
- Grunstein, M. & Hogness, D. S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3961–3965 (1975).
- Schleif, R. F. & Wensink, P. C. in *Practical Methods in Molecular Biology*, 156–159 (Springer, New York, 1981).
- Yanaihara, N. *et al. Gastroenterology* **72**, 803–810 (1977).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
- Nagata, S., Mantei, N. & Weissman, C. *Nature* **287**, 401–408 (1980).

## A major rearrangement in the *H-2* complex of mouse *t* haplotypes

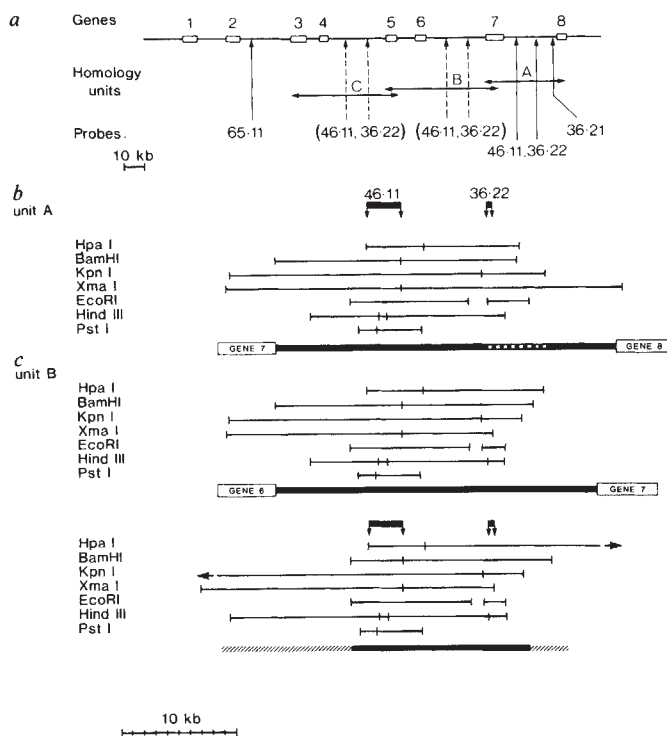
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A proportion of wild mice carry a chromosome 17 of which a large part is very different from the standard mouse chromosome 17. The affected region is called the *t* complex, and the anomalous chromosomal types are the *t* haplotypes. In combination with various other chromosomes 17, *t* haplotypes can produce crossover suppression, taillessness, transmission distortion, male sterility and lethality early in development<sup>1–3</sup>. The various *t* haplotypes also carry *H-2* specificities which are different from those of other mice<sup>3–8</sup>. This, together with the fact<sup>9,10</sup> that the lethality genes map to both sides of *H-2*, suggests that the major histocompatibility complex is contained within the *t* complex. The lack of recombination between *t* haplotypes and standard chromosomes 17 may be due to large-scale rearrangements. Genetic data support this idea<sup>8–10</sup>, in that the *tuffed* gene, the *H-2* complex and a group of *H-2*-related genes appear to be in inverted order in *t* haplotypes relative to the standard chromosome 17. The mapping of several *t*-lethal factors close to the *H-2*-related genes in *t* haplotypes<sup>9</sup> suggests that breakpoint(s) may be found here. We have now investigated the major histocompatibility complex of *t* haplotypes by Southern blots using a variety of cloned DNA probes, and find a major rearrangement, specific to the *t* haplotypes, in the *Qa-2,3* region of the complex. This involves the loss of several large homology units, probably including several class I *H-2*-related genes, and the creation of two possible breakpoints.



**Fig. 1** *a*, Map of BALB/c gene clusters 1 (genes 1–7) and 9 (gene 8) from Steinmetz *et al.*<sup>11</sup>. The overlap of these clusters around probe 36.22 was deduced from the published restriction maps<sup>11</sup> and from our genomic Southern blots, and was proved by Southern blots of cosmids 46.1 from cluster 1 and 36.2 from cluster 9 (data not shown; cosmids kindly supplied by M. Steinmetz). Indicated are class I genes, all transcribed from left to right; homology units defined by restriction map; and subcloned probes with their positions of origin (solid arrows) and major positions of cross-hybridization (dashed arrows). The probes are named after the cosmids of origin<sup>16</sup>. Probe 65.11 is a 2.1-kb *NruI*–*EcoRI* fragment, probe 46.11 is a 2.9-kb *ClaI*–*SmaI* fragment, and probe 36.21 is a 0.6-kb *Sau3AI* fragment (M. Steinmetz and L. Hood, personal communication). Probe 36.22 is a 0.44-kb *Sau3AI* fragment, its exact location established by the present experiments. *b*, Restriction map of homology units A and B in BALB/c. For each unit, the upper four enzyme maps were taken from published maps of clones<sup>11</sup> ( $\pm 1$  kb) with fragment sizes confirmed by our genomic blots. The lower three enzyme maps were established by our blots. The bar below each map indicates the extent of homology. Although the restriction maps of units A and B differ at and to the right of probe 36.22, the probe detects both units. *c*, Restriction map of the single homology unit in  $t^{w12}$ . All fragments were located by double-digest Southern blots, except for the *EcoRI* fragments, which were assumed to be in the same locations as the identical fragments in BALB/c unit B. The bar at the bottom shows the extent of homology to BALB/c unit B, with the implied breakpoints. At the left-hand breakpoint, the invariant *EcoRI* site cannot be resolved from the  $t$ -specific *BamHI* site. The large  $t$ -specific *KpnI* and *HpaI* fragments are  $\geq 30$  kb long.

The *Qa-2,3* region of the *H-2* complex contains numerous class I genes, defined by homology to the genes for known class I *H-2* antigens<sup>11–13</sup>. At least 10 of them have been cloned from the BALB/c mouse<sup>11</sup>. Their functions are unknown, but most of them are apparently expressible in transfected L cells<sup>14</sup>, and one that has been sequenced<sup>13</sup> encodes an apparently secreted class I sequence like those found in liver mRNA<sup>15</sup>. Eight of these genes were placed in two clusters numbered 1 and 9, which we find form a single continuous cluster (Fig. 1*a*). Within this cluster, the map of Steinmetz *et al.*<sup>11</sup> (Fig. 1*a,b*) reveals three segments with similar restriction maps, each extending ~30–45 kilobases (kb) and containing one or two class I genes. These segments are referred to as homology units.

To study this region, we have used intergenic probes<sup>16</sup> subcloned from the BALB/c genomic *H-2* clones of Steinmetz *et*

**Table 1** *H-2* haplotypes of mouse strains examined

| Mouse strain               | Genotype        |             | Restriction patterns |       |       |
|----------------------------|-----------------|-------------|----------------------|-------|-------|
|                            | <i>H-2</i>      | <i>Qa-2</i> | 46.11, 36.22         | 65.11 | 36.21 |
| <b>Standard:</b>           |                 |             |                      |       |       |
| BALB/c                     | <i>d</i>        | <i>a</i>    | +                    | +     | +     |
| 129                        | <i>b</i>        | <i>a</i>    | +                    | –     | +     |
| B6-Tla <sup>a</sup>        | <i>b</i>        | <i>a</i>    | +                    | –     | +     |
| AKR                        | <i>k</i>        | <i>b</i>    | +                    | +     | +     |
| SWR                        | <i>q</i>        | <i>a</i>    | +                    | +     | +     |
| <b><i>t</i> haplotype:</b> |                 |             |                      |       |       |
| $t^{w12}$                  | <i>w30(tw1)</i> | (?)         | <i>t</i>             | +     | +     |
| $t^{w5}$                   | <i>w31(tw5)</i> | (?)         | <i>t</i>             | +     | (?)   |
| $t^{w2}$                   | <i>w29(w5)</i>  | (?)         | <i>t</i> *           | +     | (?)   |
| $t^{w32}$                  | <i>w28(t12)</i> | (?)         | <i>t</i> *           | +     | (?)   |

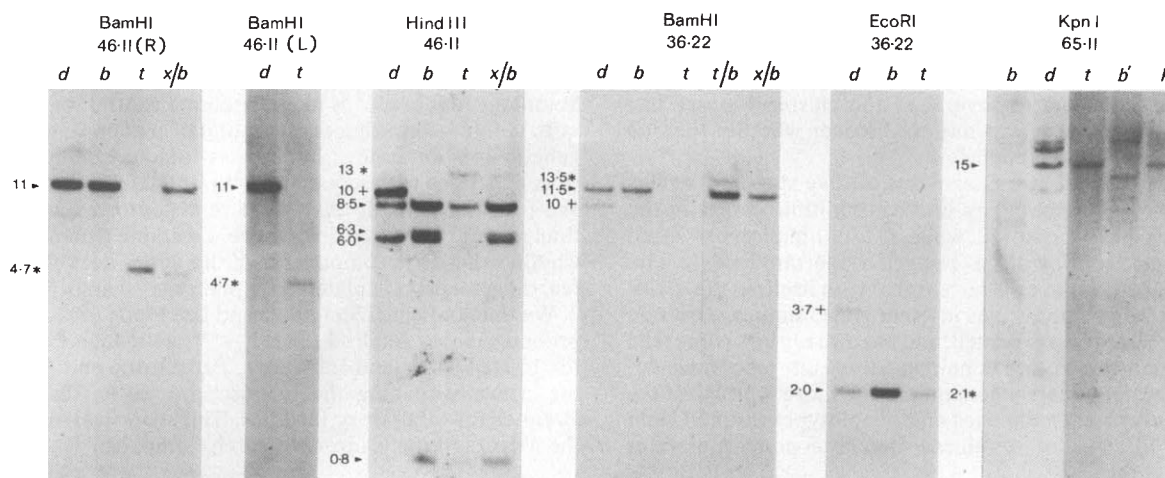
Columns 2, 3 give the known *H-2* haplotypes<sup>6</sup> and *Qa-2* alleles<sup>19</sup> of strains used (with former designations in parentheses). Columns 4–6 summarize the strain distribution of restriction patterns observed in the present experiments with the indicated probes. 46.11, 36.22: restriction patterns of homology units as described in the text (+ = standard,  $t$  =  $t$ -specific). Observed with all enzymes shown in Fig. 1 for BALB/c and  $t^{w12}$ ; with *HpaI*, *BamHI*, *EcoRI* and *KpnI* or *HindIII* for strain 129 and  $t^{w5}/129$ ; with *EcoRI* and *BamHI* for B6-Tla<sup>a</sup> and AKR; and with *BamHI* for SWR,  $t^{w2}/129$  and  $t^{w32}/BTBRTF$  (*H-2*<sup>b</sup>). *HindIII* and *EcoRI* show polymorphism among standard strains. 65.11: presence or absence of isologous band (that is, DNA identical to source of probe). Observed with *EcoRI* only for  $t^{w32}/BTBRTF$ ; with *BamHI* only for SWR and  $t^{w2}/129$ ; with *BamHI* and *KpnI* for B6-Tla<sup>a</sup> and AKR; with these plus *EcoRI* and *HindIII* for 129 and  $t^{w5}/129$ ; and with all these plus *HpaI* and *SacII* for BALB/c and  $t^{w12}/t^{w12}$ . 36.21: presence of isologous band, observed with *BamHI*. DNA for Southern blots was from liver and/or kidney of standard inbred mice (from OLAC 1976 Ltd) and of  $t^{w5}/129$ ,  $t^{w2}/129$ , and  $t^{w32}/BTBRTF$  mice (from the Pasteur Institute). For  $t^{w12}/t^{w12}$  DNA a homozygous cell line<sup>21</sup> was used. In confirmation of the cell line genotype, the  $t^{w12}$ -specific bands were seen together with strain 129 bands in Southern blots of  $t^{w12}/129$  mouse liver DNA. \*Probe 46.11 only.

*al.*<sup>11</sup>. The probes have low genomic copy numbers and so permit the specific examination of the cluster 1 homology units, which is not possible with class I gene probes because too many genes cross-hybridize. The major rearrangement to be described here is detected with probes 46.11 and 36.22. Both of these come from one of the large homology units (unit A in Fig. 1) and cross-hybridize with the other homology units.

On Southern blots of BALB/c DNA, both probes indeed detect the fragments expected from homology units A and B (Figs 1*b*, 2). It is not clear whether unit C is also detected, as most of its fragments should be identical to those of units A or B. However, we probably detect at least one additional homology unit, with a fragment pattern indistinguishable from that of unit B, which has not been identified in the cosmid clones of ref. 11. This is likely because with probe 36.22 the band corresponding to unit B is usually about twice as dark as the isologous band from unit A (marked '+' in Fig. 2). In three other standard strains of mice (Table 1), the bands corresponding to unit B are mainly identical to those in BALB/c, but the bands from unit A are absent. Homology unit A is therefore specific to BALB/c, while unit B is common to all standard strains tested.

In DNA from  $t^{w12}/t^{w12}$  cells, these sequences are substantially rearranged. With some enzymes, probes 46.11 or 36.22 detect a band of the same size as in the standard strains, but several times fainter. With other enzymes, such as *BamHI*, the band in  $t^{w12}$  is of a different size (Fig. 2). This enabled us to measure the intensity ratio of the 'standard' to the  $t$ -specific band by densitometry. The intensity ratio of the bands in *BamHI* digests of 129/ $t^{w12}$  heterozygous DNA was  $7.1 \pm 0.9$ . The ratio was also measured from parallel lanes of *EcoRI*- or *BamHI*-digested homozygous DNAs, normalized by rehybridization with a single-copy immunoglobulin C<sub>μ</sub> probe. In these experiments the 129: $t^{w12}$  ratio was  $4.8 \pm 1.3$ , and the BALB/c: $t^{w12}$  ratio was  $3.3 \pm 1.3$  (mean of three experiments





**Fig. 2** Representative Southern blots. DNA as described in Table 1 was digested with the indicated enzymes, electrophoresed on 0.7% agarose gels, blotted by the Southern<sup>21</sup> method, hybridized with the indicated probes (<sup>32</sup>P-labelled by nick translation) and washed in 2×SSC at 65 °C. Probe 46.11 was cut in two with *Pst*I to generate probes R and L. *H-2* haplotypes are abbreviated as follows: *d* (BALB/c), *b* (129), *b'* (B6-Tla<sup>a</sup>), *k* (AKR), *t* (*t*<sup>w12</sup>/*t*<sup>w12</sup>), *t/b* (*t*<sup>w12</sup>/129), *x/b* (*t*<sup>w5</sup>/129). Sizes in kb: \**t*-specific fragment, +BALB/c-specific fragment. Additional polymorphisms between *H-2*<sup>d</sup> and *H-2*<sup>b</sup> are visible in *Hind*III and *Kpn*I panels.

with each probe). As indicated below, there seems to be a single copy of the probed sequences in *t*<sup>w12</sup>; therefore, there must be three or four copies in BALB/c (consistent with the cloned BALB/c homology units described above) and four to seven copies in strain 129. The greater intensity of bands in strain 129 implies that although 129 lacks homology unit A, it contains additional copies of unit B.

One alternative explanation for the lower intensity of hybridization in *t*<sup>w12</sup> might be partial deletion of the probe sequences. This is not the case, because some enzymes produce the same-sized fragments in *t*<sup>w12</sup> and standard strains, and also because separated halves of probe 46.11 detect the same BALB/c and *t*<sup>w12</sup> bands in similar ratios (Fig. 2, first two panels). Another explanation might be general sequence divergence, but this is not the case either, as blots of *Bam*HI-digested 129/*t*<sup>w12</sup> DNA with probe 46.11 washed at various stringencies again show the same bands with the same relative intensities, although different absolute intensities. Therefore, there must be a lower copy number in the *t*<sup>w12</sup> haplotype—probably a single copy of each probe sequence, as all enzymes tested give a single restriction map (see below).

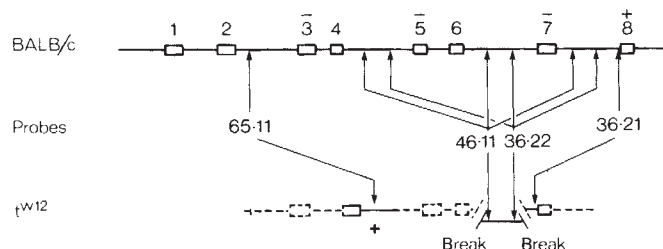
The single homology unit remaining in *t*<sup>w12</sup> differs from any seen in the five standard strains tested. However, it is also present in the *t*<sup>w5</sup>, *t*<sup>w2</sup> and *t*<sup>w32</sup> haplotypes, representing three other *t*-associated *H-2* haplotypes (Table 1). These *t* haplotypes were only available in heterozygotes with *H-2*<sup>b</sup> chromosomes, so the absence of the standard homology units could not be rigorously tested, but *Bam*HI digests of this DNA revealed the *t*-specific bands, again four- to seven-fold fainter than the standard bands. For *t*<sup>w5</sup> this was confirmed with *Hind*III (probe 46.11: Fig. 2, lanes marked 'x/b') and *Hpa*I (probe 36.22). Thus, the rearrangement of these sequences seems to be a common feature of *t* haplotypes.

The homology unit remaining in *t*<sup>w12</sup> was mapped by Southern blots of singly and doubly digested *t*<sup>w12</sup>/*t*<sup>w12</sup> DNA (Fig. 1c). The map is identical to that of BALB/c unit B for all 18 restriction sites mapped over a 15-kb region which includes both probes. It differs completely from the BALB/c maps for the four sites beyond 1.7 kb to the left of probe 46.11 and for the two sites beyond 3–4 kb to the right of probe 36.22. At these two points the cluster 1 sequences are joined to sequences of unknown origin. To summarize the analysis with probes 46.11 and 36.22, all the complete homology units of standard strains are missing from *t*<sup>w12</sup>, but the central segment of one such homology unit is present with different flanking sequences.

To examine sequences outside this segment in *t* haplotypes, we used probes 65.11 and 36.21 (Fig. 1a, Table 1). Although

each of these probes detects several bands of unknown origin in BALB/c (refs 11, 16), one of the darker bands can always be identified as the isologous band by comparison with the cluster 1 cosmid maps. In each case the isologous band is also present in *t*<sup>w12</sup>, indicating that these outer portions of cluster 1 (including most of genes 2 and 8) are unaffected by the major rearrangement. (Incidentally, probe 65.11 does show different arrangements, both among standard strains as previously noted<sup>11</sup>, and among different *t* haplotypes. Strain 129 lacks the isologous sequence, while *t*<sup>w12</sup>, *t*<sup>w2</sup> and *t*<sup>w32</sup>, but not *t*<sup>w5</sup>, have a second, variant copy of the sequence; see Fig. 2 and Table 1.) The results with all these probes are summarized in Fig. 3.

Although the probes we have used are from intergenic regions, and the remaining segment of a homology unit which we have mapped in *t*<sup>w12</sup> is entirely intergenic sequence, genes are probably included in the major rearrangement (Fig. 3). In BALB/c, each homology unit carries one or two class I genes, so the reduced copy number of the intergenic sequences in *t*<sup>w12</sup> most probably entails a reduced number of class I genes. All the class I genes in BALB/c, including those of cluster 1, can be detected with the 3' cDNA probe pH-2IIa (refs 13, 17), and the *Bam*HI fragments corresponding to each gene have been listed<sup>11</sup>. In Southern blots of *Bam*HI-digested *t*<sup>w12</sup>/*t*<sup>w12</sup> DNA probed with pH-2IIa, certain BALB/c bands are missing (data not shown, and ref. 8), and these all correspond to genes of clusters 1 and 3. From cluster 1, the 5.7-kb band corresponding to genes 3 and 5 (which is also present in *H-2*<sup>b</sup> and *H-2*<sup>a</sup>) is absent from *t*<sup>w12</sup>. (The other genes in the cluster fall in crowded regions of the blot.) Similarly, in blots of *Bam*HI-digested *t*<sup>w12</sup>/*t*<sup>w12</sup> DNA with the 5' cDNA probe pH-2III (ref. 17), the



**Fig. 3** Disposition of cluster 1 sequences in *t*<sup>w12</sup>, compared with BALB/c. Conserved sequences are split into at least three segments whose relative positions in *t*<sup>w12</sup> are unknown. Regions shown dashed have not been mapped, but fragments corresponding to at least genes 3, 5 and 7 are altered or absent in *t*<sup>w12</sup>. There is also a second, altered copy of 65.11 sequences in *t*<sup>w12</sup>.

5.9-kb band corresponding to gene 7 is absent, while the 1.4-kb band corresponding to gene 8 is retained (data not shown, and ref. 8). Thus, these patterns are consistent with (although they cannot prove) the loss of several of the cluster 1 genes in *t* haplotypes. It will be interesting to discover whether there is any associated functional defect.

In conclusion, we have shown that all five standard strains tested share similar homology units, in multiple copies in the two strains we have measured, while all four *t* haplotypes tested have a single copy with a *t*-specific rearrangement. The difference in copy number is not surprising in itself; in the major histocompatibility complex, as in other gene families, variation in copy number is to be expected, and has already been observed among mouse strains<sup>11</sup> and in human tissue culture<sup>18</sup>. However, the restriction-map rearrangement may be more unusual. If the difference between the standard and *t* haplotypes resulted from the usual processes of multigene families—point mutations

followed by gene conversion or homologous duplication/deletion—then polymorphic and monomorphic restriction sites would probably be interspersed. Instead, the remaining homology unit of *t*<sup>w12</sup> is identical to the central segment of one in BALB/c—a striking conservation of sequence in view of the genetic isolation of the *t* haplotypes—and the differences result from junctions with sequences with unrelated restriction maps. We consider it likely that these represent breakpoints rather than general sequence divergence. Genomic cloning will reveal whether these are components of the large-scale chromosomal rearrangements postulated to explain the *t* haplotypes.

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- Bennett, D. *Cell* **6**, 441–454 (1975).
- Silver, L. M. *Cell* **27**, 239–240 (1981).
- Klein, J. & Hammerberg, C. *Immun. Rev.* **33**, 70–104 (1977).
- Hammerberg, C. & Klein, J. *Nature* **258**, 296–299 (1975).
- Levinson, J. R. & McDevitt, H. O. *J. exp. Med.* **144**, 834–839 (1976).
- Sturm S., Figueroa, F. & Klein, J. *Genet. Res.* **40**, 73–88 (1982).
- Silver, L. M. *Cell* **29**, 961–968 (1982).
- Shin, H.-S., Stavnezer, J., Artzt, K. & Bennett, D. *Cell* **29**, 969–976 (1982).
- Artzt, K., McCormick, P. & Bennett, D. *Cell* **28**, 463–470 (1982).
- Artzt, K., Shin, H.-S. & Bennett, D. *Cell* **28**, 471–476 (1982).
- Steinmetz, M., Winoto, A., Minard, K. & Hood, L. *Cell* **28**, 489–498 (1982).
- Margulies, D. H., Evans, G. A., Flaherty, L. & Seidman, J. G. *Nature* **295**, 168–170 (1982).
- Steinmetz, M. *et al.* *Cell* **25**, 683–692 (1981).
- Goodenow, R. S. *et al.* *Nature* **300**, 231–237 (1982).
- Cosman, D., Khoury, G. & Jay, G. *Nature* **295**, 73–76 (1982).
- Winoto, A., Steinmetz, M. & Hood, L. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3425–3429 (1983).
- Steinmetz, M. *et al.* *Cell* **24**, 125–134 (1981).
- Gladstone, P., Fuerzes, L. & Pious, D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1235–1239 (1982).
- Flaherty, L., Zimmermann, D. & Hansen, T. H. *Immunogenetics* **6**, 245–251 (1978).
- Axelrod, H. R., Artzt, K. & Bennett, D. *Dev. Biol.* **86**, 419–425 (1981).
- Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).

## Recombination of parent and daughter strand DNA after UV-irradiation in mammalian cells

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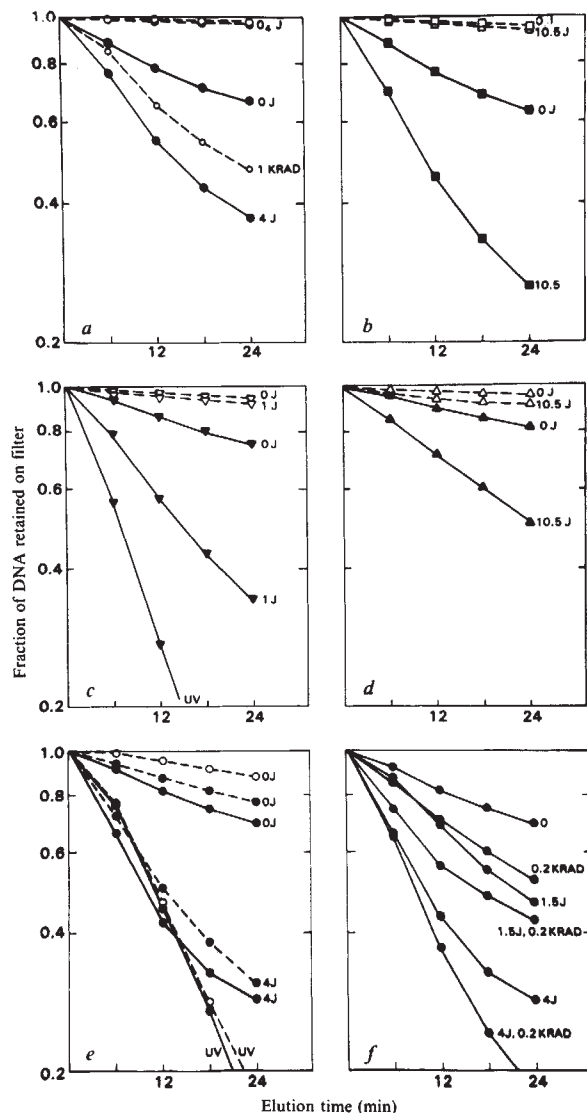
The mechanism by which mammalian cells replicate DNA containing pyrimidine dimers is poorly understood. When DNA synthesis is initiated after UV-irradiation in bacteria, parental DNA containing pyrimidine dimers has been shown to 'exchange' into the daughter strand DNA by a recA-dependent mechanism<sup>1–3</sup>. In earlier experiments, when growing mammalian cells were UV-irradiated and then incubated with labelled thymidine, pyrimidine dimers were initially interpreted to be in the newly synthesized DNA<sup>4</sup>, but later were found to be only adjacent to newly synthesized DNA in daughter strand present before UV treatment<sup>5</sup>. A way to avoid these problems of interpretation would be to use cells in G<sub>0</sub> or G<sub>1</sub> which are not synthesizing DNA at the time of irradiation. UV damage could then be detected in a very sensitive quantitative assay such as that recently described using alkaline elution and an endonuclease preparation from *Micrococcus luteus*<sup>6</sup>. I have now used this approach and report that up to 3 dimers per 10<sup>9</sup> daltons of daughter strand DNA could be detected 34–45 h after UV-irradiation in human peripheral blood lymphocytes (PBL), normal human fibroblasts, group A xeroderma pigmentosum (XP) fibroblasts and mouse 3T3 cells. This represents approximately 1–3% of the dimers present in the parent strand at this time after UV.

Freshly drawn PBL were UV-irradiated, and stimulated to enter S-phase with phytohaemagglutinin (PHA). Incorporation of <sup>14</sup>C-thymidine could only be detected 25 h or longer after addition of PHA. The radioactive thymidine (present in excess of that incorporated) was removed for the last 5 h of incubation to permit the labelled DNA replication intermediates to become large molecular weight DNA. The cell DNA was isolated on filters and the indicated samples digested with the *M. luteus* endonuclease preparation<sup>6</sup>. The rate of alkaline elution is a first order function of the frequency of DNA single-strand breaks

(SSB)<sup>7</sup>. In Fig. 1a, very little labelled DNA eluted from filters prepared either from control cells or from UV-irradiated cells when analysed by alkaline elution without prior endonuclease digestion, indicating that no SSB could be detected in these samples. When DNA was analysed from cells UV-irradiated before entry of the cells into S-phase, a large increase in elution was observed after the endonuclease digestion, indicating endonuclease-sensitive sites (ESS) in the daughter strand DNA. With DNA from unirradiated cells, only a low level of SSB was detected after endonuclease digestion, as in ref. 6. A similar effect was seen with human fibroblasts and 3T3 cells (Fig. 1b–d). These cells were grown to confluence, which concentrates human and mouse fibroblasts in G<sub>1</sub> (refs 8, 9); the cells enter S phase ~15–20 h after replating at a lower density<sup>8,9</sup>. By comparing the elution rate of samples treated as in Fig. 1 with that produced by  $\gamma$  rays, an estimate<sup>6</sup> of the frequency of ESS can be derived. In Fig. 2 with the pooled data and in individual experiments, the frequency of ESS was approximately proportional to the UV dose. Table 1 compares the frequency of ESS detected in daughter strand DNA with that present in the 'parent' strand DNA. A small fraction of the total ESS were in daughter strand DNA and it only varied by a factor of 3 in the three cell types despite very different repair capacities. DNA synthesis was >10% of control in all the determinations except XP12BE in which it was 4% (2 J m<sup>-2</sup>) and PBL in which it was 10% (10.5 J m<sup>-2</sup>).

Several objections can be raised to the interpretation that pyrimidine dimers as measured by ESS have recombined with daughter strand DNA. Could the labelled thymidine have been added to a growing DNA strand initiated before UV<sup>5</sup>. Several observations render this unlikely. It has been established that PBL are in a G<sub>0</sub> state before stimulation with a mitogen<sup>10</sup>. Human fibroblasts and 3T3 cells were confluent at the time of UV. DNA synthesis was minimal until 12–18 h after replating (Fig. 3); by 30 h after replating numerous mitoses were present. By autoradiography<sup>11</sup>, only 1% of the nuclei from confluent cells were labelled while >40% were from cells replated 24 h earlier. By fluorescence activated cell sorter (FACS-II, Becton-Dickinson), only one peak corresponding to G<sub>1</sub> cells was seen with confluent cells. Since repair synthesis is negligible in XP cells<sup>11</sup>, incorporation of label into the parent strand DNA by this mechanism can be excluded. In 1-h uptake experiments (Fig. 3), in all samples 2 mM hydroxyurea suppressed >95%

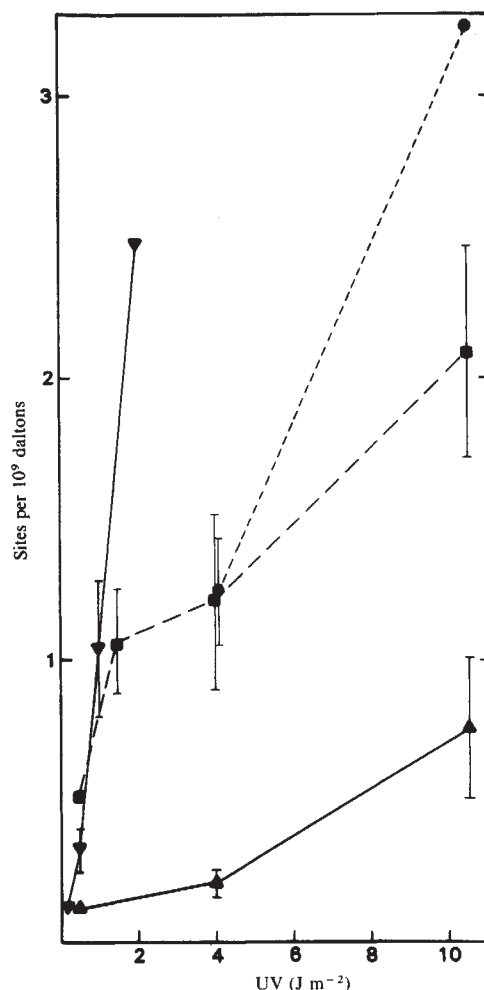




**Fig. 2** Frequency of endonuclease sensitive sites (ESS) in daughter strand DNA following UV-irradiation of stationary phase cells. Cells were treated as in Fig. 1 and the net frequency of ESS was estimated by comparing the effect with that produced by  $\gamma$ -radiation alone of L1210 cells which had been included in each experiment<sup>6</sup>. The net ESS were quantitated by (net  $e = E$  (UV, endo) -  $E$  (control, endo) -  $E$  (UV, no endo) +  $E$  (control, no endo)), where  $E = \log$  of the fraction of DNA retained on the filter after 12 min of elution, 'UV' indicates samples UV-irradiated in the stationary phase, and 'endo' samples digested with the endonuclease preparation<sup>6</sup>. The SSB frequency (ESS) was estimated by  $(2.7 \text{ per } 10^9 \text{ daltons}) \times (\text{net } e) / (E(\gamma \text{ ray, no endo}))$  where the last value represents the mean value from that experiment of L1210 cells which had been  $\gamma$ -irradiated and  $(2.7 \text{ per } 10^9 \text{ daltons})$  the SSB frequency produced by 1 krad<sup>7</sup>. The results from individual experiments were pooled and the mean is shown with the s.e.; values without the s.e. represent the mean of two or three determinations.  $\nabla$ , XP12BE;  $\blacktriangle$ , AG1518 and AG1522 (normal human fibroblasts);  $\bullet$ , PBL;  $\blacksquare$ , 3T3 cells.

**Fig. 1** Detection of *M. luteus* endonuclease-sensitive sites (ESS) in daughter strand DNA by alkaline elution. Cells were UV-irradiated with the indicated dose (J represents  $\text{J m}^{-2}$ ), then stimulated to enter DNA synthesis, labelled with  $^{14}\text{C}$ -thymidine, and analysed by alkaline elution approximately 1.5 days after UV<sup>6</sup>. The samples designated by closed symbols and solid lines were digested with an *M. luteus* endonuclease preparation before alkaline elution<sup>6</sup>. With the samples indicated by open symbols and dashed lines, the endonuclease treatment was omitted. For comparison, the effect of 1 krad of  $\gamma$  rays (3 SSB per  $10^9$  daltons)<sup>7</sup> on prelabelled cells at  $0^\circ\text{C}$  (without endonuclease) is shown in a, and  $0.2 \text{ J m}^{-2}$  (UV) on prelabelled cells in c and e. a, Peripheral blood lymphocytes (PBL); b, NIH 3T3 cells; c, xeroderma pigmentosum (XP) fibroblasts, XP12BE; d, normal human fibroblasts, AG1518; e, PBL were treated as in a with a more purified preparation of *M. luteus* UV-endonuclease (dashed lines, closed circles); f, PBL were treated as in a except the indicated samples were also  $\gamma$ -irradiated with 0.2 krad at  $0^\circ\text{C}$  immediately before analysis.

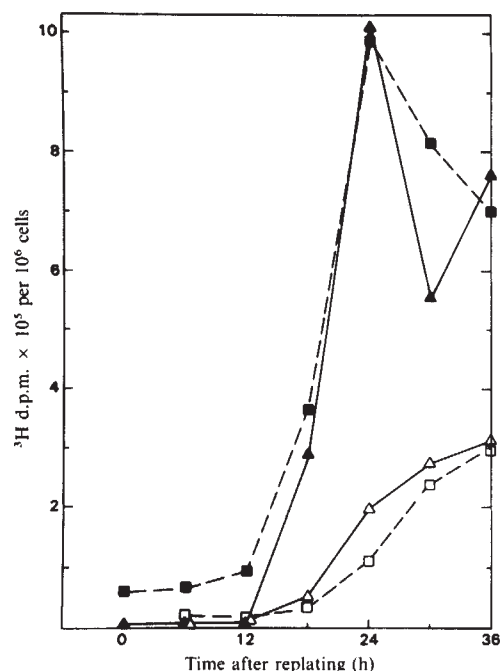
**Methods:** Cells were UV-irradiated (254 nm) as in ref. 6 except PBL which were suspended in buffered saline. After digestion with the endonuclease preparation ( $0.17 \text{ mg ml}^{-1}$  protein of fraction II)<sup>23</sup> for 1 h, alkaline elution was carried out as previously described at  $0.7 \text{ ml min}^{-1}$  (ref. 6). The more purified preparation in e was provided by Jay Doniger and represented peak II from the P-11 column of ref. 14; this was diluted 1:10 in the standard buffer<sup>6</sup> except at pH 7.5 with  $0.5 \text{ mg ml}^{-1}$  albumen without carrier DNA; incubation was also carried out with this buffer without enzyme in e (dashed line, open circles). PBL were isolated from freshly drawn blood of a normal 33-yr-old male by Ficoll-Hypaque sedimentation, rinsed, UV-irradiated and grown in RPMI 1640 medium, 10% heat inactivated fetal calf serum, penicillin/streptomycin, and  $2 \mu\text{g ml}^{-1}$  phytohaemagglutinin for 47 h; with  $^{14}\text{C}$ -thymidine,  $0.06 \mu\text{Ci ml}^{-1}$ ,  $0.3 \mu\text{M}$ , from 26 to 42 h after UV. Confluent fibroblasts (see Fig. 3) were UV-irradiated, trypsinized and seeded at a lower density for 35–36 h with  $^{14}\text{C}$ -thymidine,  $0.03 \mu\text{Ci ml}^{-1}$ ,  $0.6 \mu\text{M}$ , from 19 to 29 h after UV.



**Table 1** Estimated frequency of endonuclease-sensitive site (ESS) exchanges

| Cell type | Fraction of ESS remaining in parent strands after 35 h (dose, $\text{J m}^{-2}$ ) | ESS in daughter strand per $10^9$ daltons (dose, $\text{J m}^{-2}$ ) | Daughter strand ESS/parent strand ESS | DNA synthesis (% of control) |
|-----------|-----------------------------------------------------------------------------------|----------------------------------------------------------------------|---------------------------------------|------------------------------|
| AG1522    | 0.14 (1.0)                                                                        | 0.2 (4)                                                              | 0.012                                 | 60                           |
| XP12BE    | 0.96 (0.2)                                                                        | 1.0 (1)                                                              | 0.036                                 | 19                           |
| 3T3       | 0.62 (0.5)                                                                        | 1.2 (4)                                                              | 0.016                                 | 86                           |

The fraction of ESS remaining in parent strand DNA after approximately 35 h of repair incubation was determined by experiments as in Fig. 1 except the cells were prelabelled with  $^{14}\text{C}$ -thymidine. ESS in daughter strand DNA is taken from Fig. 2. 'ESS in daughter strand/ESS in parent strands' represents the value from the second column divided by the estimated frequency of ESS remaining in the parent strand DNA at this time - initial dose  $\times$  fraction remaining (col. 1)  $\times 30$  ESS per  $10^9$  daltons per  $\text{J m}^{-2}$ , since the fraction of ESS removed during a particular repair time is constant with doses up to at least  $4 \text{ J m}^{-2}$  (refs 6, 22). In the last column, DNA synthesis (expressed as percentage of unirradiated) is shown with the same dose as in the column designated 'ESS in daughter strand per  $10^9$  daltons'. It was assumed that all the daughter strand ESS were detected in experiments in Figs 1 and 2. It has previously been shown that the endonuclease reaction went to completion with doses up to  $1 \text{ J m}^{-2}$  (ref. 6). Also, when PBL were assayed as in Fig. 1a with twice the endonuclease concentration, the same frequency of ESS (net  $e$ ) was detected.



**Fig. 3** DNA synthesis. Incorporation of  $^3\text{H}$ -thymidine in normal human fibroblasts, AG1522 (▲) and 3T3 cells (■) was determined after replating the cells at a lower density. The values at 0 h represent incorporation into confluent cells. Open symbols represent samples which were irradiated with  $10.5 \text{ J m}^{-2}$  at the time of replating. Similar results were observed with AG1518 (data not shown) as with AG1522.

**Methods:** AG1522 (Camden Cell Repository) were grown to confluence in Ham's F12 medium containing 10% fetal calf serum, 17 mM HEPES buffer, 3 mM glutamine,  $20 \text{ ng ml}^{-1}$  epidermal growth factor,  $0.3 \mu\text{M}$  hydrocortisone,  $10 \mu\text{g ml}^{-1}$  gentamycin and  $\text{NaHCO}_3$  adjusted to  $1.7 \text{ mg ml}^{-1}$ . The medium was then switched for 3 days to Ham's F12 with 1% heat-inactivated fetal calf serum and the above concentration of HEPES,  $\text{NaHCO}_3$  and gentamycin. The cells were UV-irradiated (where indicated), trypsinized and seeded at a lower density in the first medium. 3T3 cells were grown to confluence in Dulbecco's minimal essential medium with 10% heat-inactivated calf serum, 17 mM HEPES, 3 mM glutamine and gentamycin; switched to the same medium but with 1% serum without glutamine for 3 days, and handled as above. Aliquots of the cells were incubated at various times for 6 h with  $1 \mu\text{Ci ml}^{-1}$   $^3\text{H}$ -thymidine,  $3 \mu\text{M}$  in AG1522 and  $1 \mu\text{M}$  in 3T3, and the number of acid precipitable d.p.m. of  $^3\text{H}$  determined.

of  $^3\text{H}$ -thymidine incorporation into AG1522 and 3T3 cells when added 24 h after replating.

Another possibility may be that the rapidly eluting unlabelled parent strand affected the elution rate of the daughter strand. However, a dose response for ESS in daughter DNA was seen with UV doses at which all the parent strand DNA eluted very rapidly. The increase in elution on a first order plot (Fig. 1f) with a small dose of  $\gamma$  rays on daughter strand DNA was the same in control cells and cells previously UV-irradiated. The only known substrates for this endonuclease preparation in EDTA are pyrimidine dimers, apurinic sites, 3-methyladenine and ionizing radiation base damage<sup>12,13</sup>. In Fig. 1e, the same frequency of ESS in daughter strand DNA was seen with a more purified preparation of *M. luteus* UV-endonuclease (specific activity for pyrimidine dimers >50-fold of crude extract, nonspecific endonuclease activity <20-fold of crude extract<sup>14</sup>). The same level of ESS was observed with either preparation in cells labelled before UV, thus indicating that only pyrimidine dimers were recognized.

The exchange of pyrimidine dimers between parent and daughter strand DNA in bacteria is mediated by general recombination involving strand exchange between sister duplexes (discussed in refs 2, 3). In mammalian cells, a similar mechanism would explain the present results. Evidence exists for recombination—for example, sister chromatid exchanges on a cytogenetic level<sup>15</sup>, structures consistent with Holliday-type<sup>16</sup> intermediates in virus-infected HeLa cells<sup>17</sup>, and recombination of plasmids after transfection in rodent cells<sup>18</sup>. If the single-strand size of an average human chromosome is  $\sim 2 \times 10^{10}$  daltons, the frequency of ESS in Table 1 would be the same order of magnitude as the expected size of sister chromatid exchanges, although probably more frequent. If double-strand exchange is equivalent to sister chromatid exchange<sup>15</sup>, this would indicate that a major portion of the ESS detected in daughter DNA was produced by resolution of a Holliday intermediate by exchange of a limited length of single-strand DNA containing the dimer (see Fig. 1 of ref. 3). Various recombination intermediates or combination of intermediates could be proposed to explain such results<sup>2,3,16,19,20</sup>. Since very little, if any, DNA can be detected by density shift experiments to exchange between parent and daughter strands in mammalian cells compared with bacteria<sup>21</sup>, a reasonable interpretation is that relatively short segments of DNA are exchanged and that occasionally parent DNA containing a dimer remains in the daughter strand.

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- Rupp, W. P., Wilde, C. E., Reno, D. L. & Howard-Flanders, P. *J. molec. Biol.* **61**, 25–44 (1971).
- Hanawalt, P. C., Cooper, P. K., Ganesan, A. K. & Smith, C. A. *A. Rev. Biochem.* **48**, 783–836 (1979).
- Dressler, D. & Potter, H. *A. Rev. Biochem.* **51**, 727–761 (1982).
- Buhl, S. N. & Regan, J. D. *Nature* **246**, 484 (1973).
- Lehmann, A. R. & Kirk-Bell, S. *Photochem. Photobiol.* **27**, 297–307 (1978).
- Fornace, A. J. Jr *Mutat. Res.* **94**, 263–276 (1982).
- Kohn, K. W., Erickson, L. C., Ewig, R. A. G. & Friedman, C. A. *Biochemistry* **15**, 4628–4637 (1976).
- Nagasawa, H., Robertson, J. & Little, J. B. *Radiat. Res.* **83**, 368–369 (1980).
- Nagasawa, H. & Little, J. B. *Radiat. Res.* **91**, 301 (1982).
- Darzynkiewicz, Z., Traganos, F., Sharpless, T. & Melamed, M. R. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2881–2884 (1976).
- Robbins, J. H. *Ann. intern. Med.* **80**, 221–248 (1974).
- Paterson, M. C. *Adv. Radiat. Biol.* **7**, 1–53 (1978).
- Laval, J. *Nature* **269**, 829–832 (1977).
- Riazuddin, S. & Grossman, L. *J. biol. Chem.* **252**, 6280–6286 (1977).
- Latt, S. A. *A. Rev. Genet.* **15**, 11–55 (1981).
- Holliday, R. *Genet. Res., Camb.* **5**, 282–304 (1964).
- Wolgemuth, D. J. & Hsu, M. *Nature* **287**, 168–170 (1980).
- Small, J. & Scangos, G. *Science* **219**, 174–176 (1983).
- Lavin, M. F. *Biophys. J.* **23**, 247–255 (1978).
- Cunningham, R. P., Wu, A. M., Shibata, T., DasGupta, C. & Radding, C. M. *Cell* **24**, 213–223 (1981).
- Loveday, K. S. & Latt, S. A. *Nucleic Acid Res.* **5**, 4087–4104 (1978).
- Kantor, G. J. & Setlow, R. B. *Cancer Res.* **41**, 819–825 (1981).
- Carrier, W. L. & Setlow, R. B. *J. Bact.* **102**, 178–186 (1970).



# Association of an S<sub>1</sub> nuclease-sensitive structure with short direct repeats 5' of *Drosophila* heat shock genes

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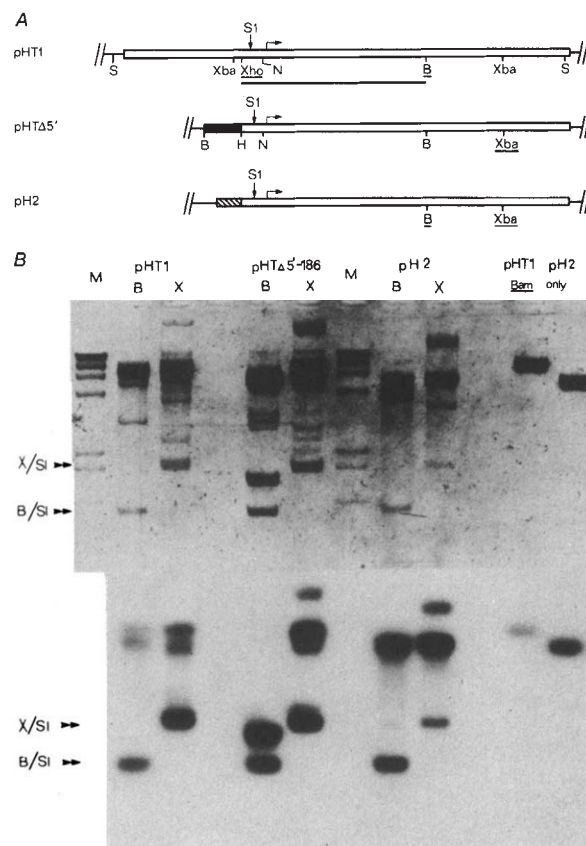
The potential for a DNA molecule to assume different tertiary structures has been suggested as a possible determinant of transcriptional regulation in eukaryotes<sup>1,2</sup>. Such forms include cruciforms<sup>3,4</sup> transitions from B to Z DNA in the same molecule<sup>2,5</sup> and short unpaired stretches of DNA in an alternating copolymer<sup>6</sup>. All such structures contain regions which are preferentially sensitive to S<sub>1</sub> nuclease<sup>4-6</sup>. We report here an additional class of S<sub>1</sub> nuclease sensitive sites associated with short direct repeats of DNA found in the 5' flanking regions of certain *Drosophila melanogaster* heat shock genes.

The DNA sequences specifying heat inducible regulation of the major *Drosophila* heat shock gene, *hsp70*, have been precisely identified<sup>7-9</sup>. To characterize other features of the 5' flanking region which might be involved in expression we examined three plasmids containing this gene for S<sub>1</sub>-sensitive sites. The structures of these plasmids have been described previously, and are outlined in Fig. 1. Plasmid pHT1<sup>7</sup> contains an entire *hsp70* coding region and 1,140 base pairs (bp) of 5' flanking sequence derived from the 87C chromosomal locus<sup>10</sup>, while plasmid pHTΔ5'-186<sup>7</sup> contains only 186 bp of 5' flanking sequence, derived from a copy of the *hsp70* gene at chromosomal locus 87A<sup>10</sup>. The third plasmid pH2<sup>7</sup> contains the simian virus 40 (SV40) origin region juxtaposed to the 5' side of the *hsp70* gene (from 87C), about 190 bp from the transcription start site. As a consequence *hsp70* RNA is expressed constitutively *in vivo*<sup>7</sup> from pH2.

Treatment of these supercoiled plasmids with S<sub>1</sub> nuclease resulted in their rapid conversion to nicked circular DNA which in turn was slowly converted to the linear form (data not shown). Subsequent digestion of the linearized plasmids with *Bam*HI or *Xba*I yielded a number of discrete fragments in addition to those obtained by restriction alone (Fig. 1B, upper panel), indicating that S<sub>1</sub> cuts at a small number of specific sites. This specific cutting did not occur if the supercoiled plasmids were restricted before S<sub>1</sub> digestion (data not shown).

The DNA fragments produced by S<sub>1</sub> and *Bam*HI or *Xba*I were transferred to nitrocellulose<sup>11</sup> and hybridized to a probe which extended from the *Xho*I site at -190 to the *Bam*HI site at position +1,265 of the *hsp70* gene (Fig. 1B, lower panel). With each of the three plasmids this probe hybridized strongly to a 1,400 bp S<sub>1</sub>/*Bam*HI fragment and a 2,000 bp S<sub>1</sub>/*Xba*I fragment. This is consistent with cleavage by S<sub>1</sub> at about position -140 of the *hsp70* gene (Fig. 1A). The other hybridizing bands in Fig. 1B are derived from plasmid that was not cut by S<sub>1</sub> nuclease. Ethidium bromide staining of the digests indicated that the *hsp70* gene contained the preferred site of S<sub>1</sub> cleavage, the percentage of plasmid molecules being cut at this site varying from 20% to as much as 50% in some experiments.

To map the *hsp70* S<sub>1</sub> cleavage site more precisely pHTΔ5'-186 was cleaved with S<sub>1</sub> nuclease, then with *Bam*HI, and the S<sub>1</sub> generated fragments isolated. These fragments were then terminally labelled and further restricted with endonucleases which cleave close to the assumed position of the site. The resulting small fragments were sized precisely on a sequencing gel (Fig. 2) which showed that the major termini generated by S<sub>1</sub> nuclease activity centred closely around position -124 (Fig. 4). By contrast when pHT1 was analysed in a similar manner a different pattern of termini was observed. In this case the

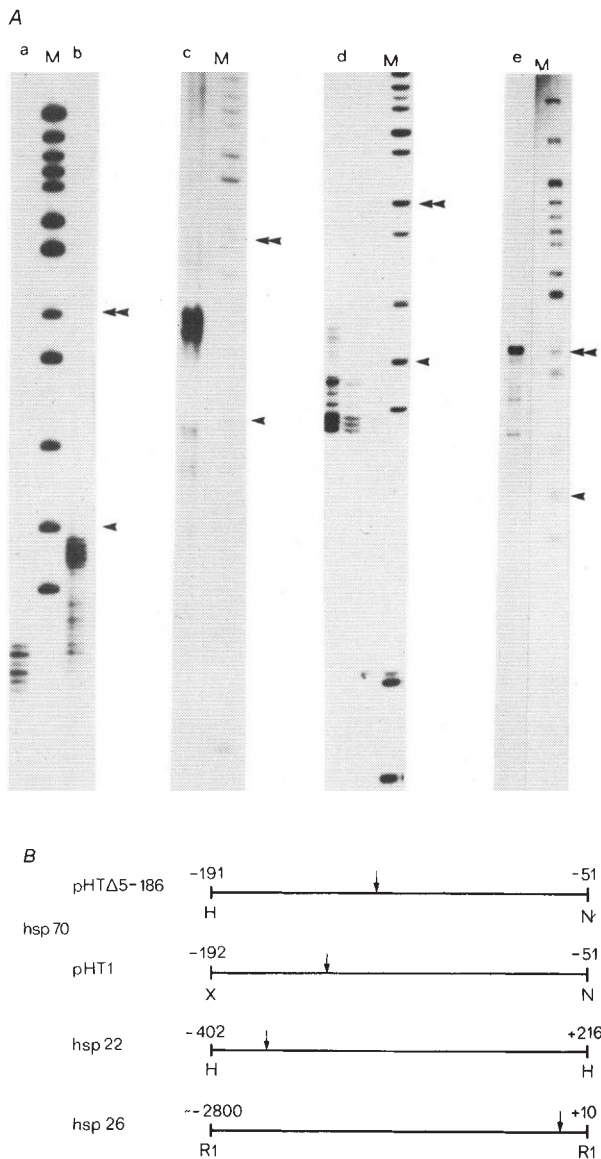


**Fig. 1.** A, Restriction maps of the relevant regions of plasmids pHT1, pHTΔ5'-186 and pH2. B, Analysis of S<sub>1</sub> nuclease-treated plasmids.

**Methods:** A, The plasmids are fully described in ref. 7. Unique restriction sites are underlined; B, H, N, S, Xba, Xho, indicate *Bam*HI *Hind*III, *Nru*I, *Sal*I, *Xba*I and *Xho*I sites respectively. The *Bam*HI-*Xho*I fragment from pHT1 used to probe the restriction digests is shown. Clear boxes indicate *hsp70* sequences; black, 5S spacer DNA; diagonal stripes, SV40 DNA sequences,  $\rightarrow$  indicates transcription startpoint. B, pHT1, pHTΔ5'-186 and pH2 were treated with S<sub>1</sub> nuclease. The reaction mixture (100  $\mu$ l) contained ~2.5  $\mu$ l supercoiled plasmid DNA, 700 units S<sub>1</sub> nuclease (Boehringer), 40 mM sodium acetate, pH 4.5, 50 mM sodium chloride and 1 mM zinc chloride, and was incubated for 10 min at 37 °C. The treated DNA was then extracted with phenol, spun through a small Sephadex G-50 column and subsequently digested with either *Bam*HI or *Xba*I. The digests were electrophoresed on a 1% agarose gel and then probed with the *Xho*I-*Bam*HI fragment from pHT1 by the method of Southern<sup>11</sup>. The digests are displayed as visualized by ethidium bromide staining (upper panel) or by Southern blotting (lower panel). For each plasmid; track B, S<sub>1</sub> nuclease followed by *Bam*HI digestion; track X, S<sub>1</sub> nuclease followed by *Xba*I digestion; track Bam only, *Bam*HI digestion only. Note that pHT1 has two *Xba*I sites and that the major S<sub>1</sub>-*Xba*I fragment is poorly separated from the shorter *Xba*I-*Xba*I fragment. Similarly pHTΔ5'-186 has two *Bam*HI sites. B/S<sub>1</sub> and X/S<sub>1</sub> indicate the S<sub>1</sub>-*Bam*HI and S<sub>1</sub>-*Xba*I fragments respectively containing the *hsp70* transcription start point. M is a *Hind*III, *Kpn*I mixed digest of  $\lambda$  DNA with visible fragments of sizes ~21,694, 9,539, 6,602, 4,601, 2,320, 2,002 and 1,503 bp.

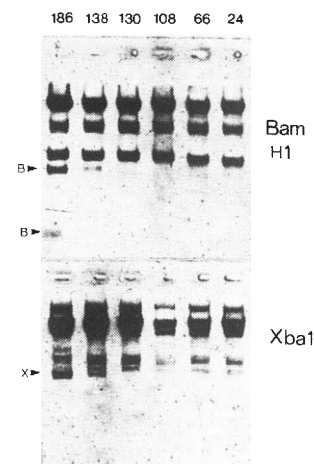
most abundant termini were centred at ~-145 while a second minor set were observed at similar positions to those characterized in pHTΔ5'-186.

The extent of the DNA sequences necessary to define the S<sub>1</sub> nuclease sensitive site was investigated by analysis of a set of plasmids homologous to pHTΔ5'-186 in which the 5' flanking region of *hsp70* was progressively deleted<sup>7</sup>. When the *hsp70* sequence extended to position -138 the site was still present but the proportion of plasmid DNA cleaved at this site was reduced about fivefold (Fig. 3). By contrast S<sub>1</sub> cleavage of



**Fig. 2** A, Mapping of  $S_1$  sites. B, Maps of sites used in each plasmid.

**Methods:** A, pHTΔ5'-186 was treated with  $S_1$  nuclease and the cut plasmid terminally labelled at the  $S_1$  site with polynucleotide kinase. The labelled plasmid was then digested with *Hind*III (track a) or *Nru*I (track b) and the labelled fragments analysed by polyacrylamide gel electrophoresis. Track c shows a comparable  $S_1$ -*Nru*I digest from pHT1. Track d maps the  $S_1$  site in the *hsp22* promoter. The plasmid pT6BV1, containing the *hsp22* gene, was cut with  $S_1$  nuclease; then with *Hind*III and the resulting digest terminally labelled by filling in 3' termini and the labelled fragments analysed. Track e maps the  $S_1$  site in the *hsp26* promoter. In this case an *Eco*RI fragment containing the promoter was inserted in a vector containing SV40 and thymidine kinase sequences<sup>16</sup>. This plasmid was digested with  $S_1$  nuclease, then with *Eco*RI and the digest terminally labelled as for *hsp22*. For both *hsp22* and *hsp26* the approximate location of the  $S_1$  site was initially established by analysis with different restriction enzymes. B, H, N, R1, X indicate *Hind*III, *Nru*I, *Eco*RI and *Xho*I sites respectively. The approximate locations of the  $S_1$  nuclease sites are indicated by arrows. The numbering convention adopted for pHT1 and pHTΔ5'-186 is to include the first three bases of the restriction site and for *hsp22* and *hsp26* to indicate the extent of the filled in fragment. The data for mapping the  $S_1$  cuts in pHT1 from the *Xho*I site are not shown. The marker lanes contain a terminally labelled *Msp*I digest of pBR322 with visible fragments of sizes 311, 244, 240, 219, 203, 192, 182, 162, 149, 124, 112, 92, 78, 69 and 36 bp. The 124 and 78 bp fragments are indicated by double and single arrows respectively.



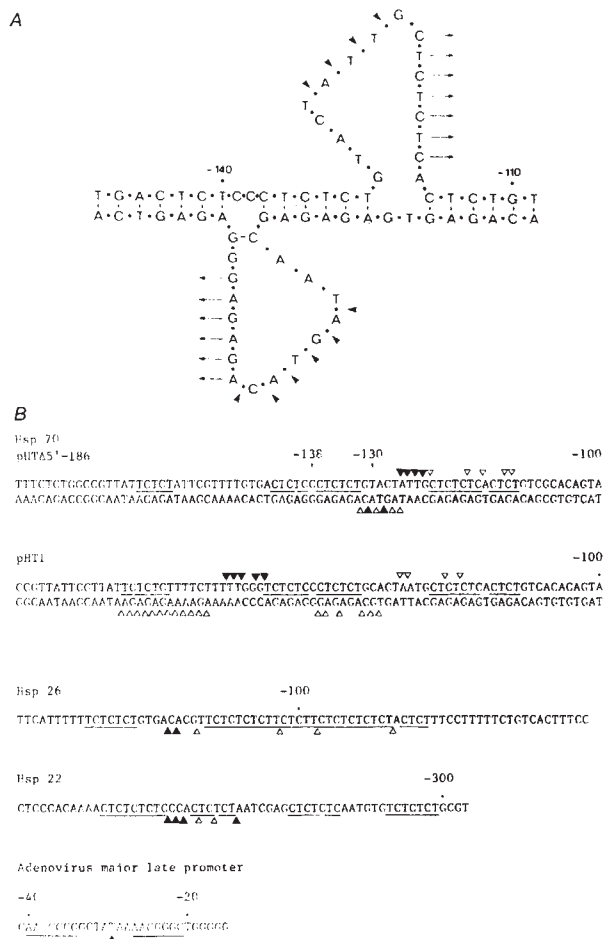
**Fig. 3** Deletion analysis of  $S_1$  cleavage site in pHTΔ5'-186. Plasmids with 186, 138, 108, 66 and 28 bp of 5' flanking *hsp70* sequence were treated with  $S_1$  nuclease and then with *Bam*HI or *Xba*I. The resulting fragments were analysed by ethidium bromide staining of an agarose gel. The  $S_1$ -*Bam*HI and  $S_1$ -*Xba*I fragments arising from cleavage upstream of the *hsp70* promoter are marked B and X respectively. The upper bands of the marker track are from the mixed *Hind*III, *Kpn*I digests of λ DNA. Note in the *Bam*HI digest of pHTΔ5'-186 a ~375 bp band (B') which is the second *Bam*HI- $S_1$  product containing sequences extending upstream of the  $S_1$  cleavage site (see Fig. 1A).

constructions with 130, 108, 66 and 28 bp of 5' flanking sequence showed no cleavage in this region. We conclude that a sequence necessary to define the  $S_1$  cleavage site occurs between -130 and -138. In addition the reduced frequency of cleavage in the -138 construction relative to the -186 construction indicates that a sequence upstream of -138 influences the proportion of plasmid molecules containing the  $S_1$  cleavage site.

The DNA sequence in the proximity of the  $S_1$  cleavage sites in pHT1 and pHTΔ5'-186 contains no inverted repeats with the potential for cruciform formation. Instead two repeats of the hexanucleotide (CT)<sub>3</sub> occur both in pHTΔ5'-186 and pHT1 centred at -127 while pHT1 has an additional repeat of (TC)<sub>3</sub> centred at ~-145<sup>12</sup>. Considering first the simpler case of pHTΔ5'-186, the major  $S_1$  generated termini occur between the elements of the direct repeat, whichever side of the cut they are mapped from. The direct repeats themselves are free of major cleavages. We propose that this pattern of termini could be generated from a structure in which the sequence (CT)<sub>3</sub> at -137 to -132 in the upper strand pairs with (GA)<sub>3</sub> at -122 to -117 in the lower strand (Fig. 4). Such a pairing would generate two single-stranded loops which themselves would have the potential to pair with each other utilizing the complements of these same sequences. The pattern of  $S_1$  generated termini on pHT1 may be qualitatively interpreted in a similar way. The two direct repeats of (TC)<sub>3</sub> and (CT)<sub>3</sub> would each generate a cutting site, the upstream repeat being dominant. The precise sizes of the fragments mapped may also, of course, be influenced by any 'nibbling' activity of the  $S_1$  nuclease.

Sequences similar to those that are cleaved in the *hsp70* genes occur in the 5' flanking regions of the *hsp22* and *hsp26* genes<sup>13</sup>, and we have also investigated the  $S_1$  sensitivity of these regions. Fragments containing the promoter regions of all four small heat shock genes (*hsp22*, *hsp23*, *hsp26*, *hsp27*) were subcloned, and  $S_1$  sensitive sites mapped first by restriction analysis and then by end-labelling as described for *hsp70* (Fig. 2). Neither the *hsp23* nor the *hsp27* gene, both of which lack CT-rich regions, contained a strong  $S_1$ -sensitive site, even though *hsp23* contains an extended alternating purine-pyrimidine sequence from -67 to -84 which might have the potential to form Z-DNA. However, major cleavage sites were





**Fig. 4** **A**, Possible tertiary structure of DNA at the S<sub>1</sub> nuclease site in pHTΔ5'-186. Arrows indicate possible interactions between loops. **B**, Sequences of *hsp70* (pHTΔ5'-186 and pHT1 clones), *hsp22* and *hsp26* genes showing location of major S<sub>1</sub> nuclease generated termini. CT rich sequences are underlined. Also shown is the direct repeat flanking the major S<sub>1</sub> site in the adenovirus major late promoter; the approximate cleavage site is taken from ref. 14. ▲ and △ indicate major and minor S<sub>1</sub> nuclease-generated termini. For *hsp70* the termini on each strand (that is mapped from each side of the cut) are indicated, for *hsp22* and *hsp26* no distinction is made. Note that the termini mapped do not necessarily correspond to the initial sites of S<sub>1</sub> cleavage; some may be generated by subsequent 'nibbling'.

readily detected for both *hsp22* and *hsp26* and mapped to a sequence flanked on each side by at least 6 bp of alternating CT residues (Figs 2, 4). In *hsp26* minor cleavage sites mark discontinuities in a long run of repeated CT residues.

Why should these sequences form S<sub>1</sub> nuclease sensitive structures more readily than other regions in the plasmids? pHT1 contains many short direct repeats, particularly in the very AT-rich region further upstream of the *hsp70* gene. One factor may be that the striking purine/pyrimidine asymmetry in the sequences around the S<sub>1</sub> sites contributes to some distortion or instability of the helix. Interestingly the S<sub>1</sub>-sensitive site in the TATA box of the adenovirus major late promoter<sup>14</sup> also lies within a region of very high purine/pyrimidine asymmetry and is flanked by direct repeats of the sequence AAGGGGG, suggesting that a structure similar to that proposed for the heat shock genes could form in preference to a cruciform.

An additional possibility is that the repeated dinucleotides within the direct repeats themselves may facilitate slippage and thus favour the formation of these particular S<sub>1</sub>-sensitive structures, as proposed for such sequences in the flanking regions of sea urchin histone genes<sup>6</sup>. Such slippage might be facilitated by the low pH of the S<sub>1</sub> nuclease reaction mixture which would

permit protonation of cytosine residues with a consequent weakening of G·C base pairing.

Although S<sub>1</sub>-sensitive sites are of widespread occurrence in the 5' flanking regions of eukaryotic genes the sites that have been mapped do not correspond to known promoter elements with the exception of the TATA box site in the adenovirus major late promoter<sup>14</sup>. In the case of the *hsp70* gene, deletion of the S<sub>1</sub>-sensitive region does not prevent heat-inducible transcription in either *Xenopus* oocytes<sup>15</sup> or monkey cells<sup>7</sup>, and plasmid pH2, which is expressed constitutively in monkey COS cells<sup>7</sup>, shows the same S<sub>1</sub> site as inducible copies of the gene. This does not rule out a function for the site; the genes have been assayed under conditions where there are very many copies of the gene per cell, and sequences that appear redundant under these circumstances may play a more significant role for the small number of genomic copies of the genes, for example by affecting local chromatin organization<sup>17</sup>.

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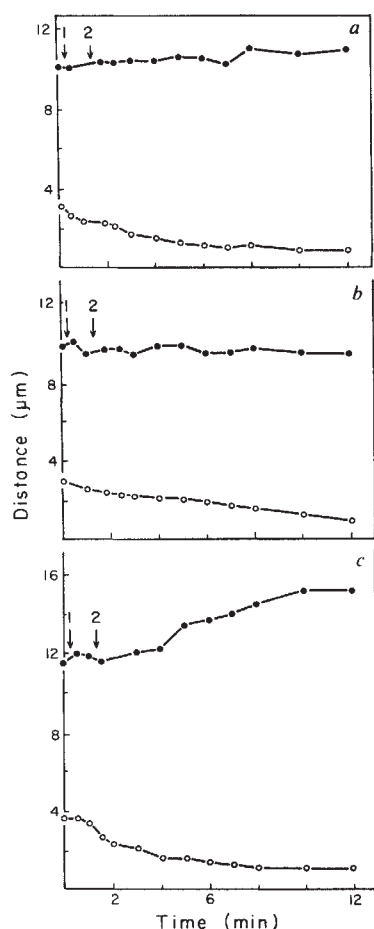
1. Larsen, A. & Weintraub, H. *Cell* **29**, 609-622 (1982).
2. Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C. & Stollar, B. D. *Cell* **31**, 309-318 (1982).
3. Lilley, D. M. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6468-6472 (1980).
4. Panayotatos, N. & Wells, R. D. *Nature* **289**, 466-470 (1981).
5. Klysik, J., Stirdivant, S. M., Larson, J. E., Hart, P. A. & Wells, R. D. *Nature* **290**, 672-677 (1981).
6. Hentschel, C. C. *Nature* **295**, 714-716 (1982).
7. Pelham, H. R. B. *Cell* **30**, 517-528 (1982).
8. Pelham, H. R. B. & Bienz, M. *EMBO J.* **1**, 1473-1477 (1982).
9. Mirault, M.-E., Southgate, R. & Delwart, E. *EMBO J.* **1**, 1279-1286 (1982).
10. Ish-Horowitz, D., Pinchin, S. M., Schedl, P., Artavanis-Tsakonas, S. & Mirault, M.-E. *Cell* **18**, 1351-1358 (1979).
11. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
12. Karch, F., Török, I. & Tissieres, A. *J. molec. Biol.* **148**, 219-230 (1981).
13. Ingolia, T. D. & Craig, E. A. *Nucleic Acids Res.* **9**, 1627-1641.
14. Golding, C. R. & Russell, W. C. *Nucleic Acids Res.* **11**, 21-36 (1983).
15. Bienz, M. & Pelham, H. R. B. *EMBO J.* **1**, 1583-1588 (1982).
16. Pelham, H. R. B. & Lewis, M. J. in *Gene Expression* (eds Hamer, D. & Rosenberg, M.) (Liss, New York, in the press).
17. Weintraub, H. *Cell* **32**, 1191-1203 (1983).

## Creatine kinase role in anaphase chromosome movement

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Creatine kinase catalyses the conversion of ADP and phosphoryl creatine into ATP and creatine. In skeletal muscle a non-mitochondrial isoenzyme of creatine kinase has a major role in regulating the energy supply for muscle contraction by maintaining the ATP pools at relatively constant levels<sup>1,2</sup>. Recently, this enzyme has been localized by immunofluorescence techniques on intermediate filaments and stress fibres in interphase cells<sup>3</sup> and in mitotic spindles of dividing tissue culture cell lines, including the PtK<sub>1</sub> cell line<sup>4,5</sup>, and has been found to co-purify with spindles isolated from dividing sea urchin eggs<sup>6</sup> and with muscle cell nuclei<sup>7</sup>. Here I have used permeabilized PtK<sub>1</sub> cells to investigate the possibility that creatine kinase may be involved in regulation of spindle energy metabolism by lysing cells in medium containing phosphoryl creatine. I find that spindle elongation will continue in these conditions, provided ADP is also present in the lysis medium. This finding is consistent with my previous demonstration that spindle elongation is the ATP-requiring step during anaphase<sup>8,9</sup> and is a direct demonstration that creatine kinase may have a role in mitosis analogous to that observed during skeletal muscle contraction.



**Fig. 1** Effect of medium composition on chromosome movements in permeabilized cells. Separation of spindle poles (●) and chromosome to pole movement (○) in one half-spindle when cells are lysed in a medium containing 1.25 mM ADP (a), 5 mM phosphoryl creatine (b) or 3 mM ADP and 5 mM phosphoryl creatine (c). PtK<sub>1</sub> cells were used in all experiments and handled for light microscopy as described in refs 8 and 10. Anaphase cells were lysed by flushing solutions under the coverslip in a two-step lysis procedure. In step 1 (arrow) the medium contained 85 mM PIPES, pH 6.94, 0.08% w/v Brij 58, nucleotide,  $10^{-7}$  M free  $\text{Ca}^{2+}$  (buffered with EGTA) and 1 mM free  $\text{Mg}^{2+}$ . A similar medium was used in step 2 (arrow), applied 70–90 s after step 1, which included in addition 2.5% w/v polyethylene glycol (molecular weight 20,000) and 0.1% w/v Brij 58.

Mitotic PtK<sub>1</sub> cells (a kidney epithelial line derived from the rat kangaroo) continue to undergo anaphase after permeabilization of the cell with the nonionic detergent Brij 58 (Fig. 1, step 1 and refs 8, 9) provided polyethylene glycol is added to the medium 1–2 min after lysis (Fig. 1, step 2). After lysis these cells are permeable to small molecules such as ions and nucleotides and to some proteins, yet 80–90% of the cell's proteins are retained in the cell model<sup>10</sup>. The rate and extent of spindle elongation (anaphase B) in lysed mitotic cells (Fig. 1) are critically dependent on the ATP concentration in the medium over a concentration range of 0.2–5 mM ATP. The rate observed in 1.25 mM ATP (Table 1) is ~20% that observed *in vivo*<sup>8</sup>. The rate and extent of chromosome to pole movement (anaphase A) after lysis (Fig. 1) are ~30% of those observed *in vivo* and are not affected by addition of nucleotides (Table 1 and ref. 8). Spindle elongation is inhibited by the addition of nonspecific and specific ATPase poisons such as *N*-ethylmaleimide, salyrgan, vanadate and EHNA, which are known to inhibit the flagellar ATPase dynein, but chromosome to pole movement is not affected by these inhibitors<sup>8,9</sup>.

**Table 1** Nucleotide dependence of chromosome movement

| Treatment              | No. of Experiments | Rates of chromosome movement ( $\mu\text{m min}^{-1}$ ) |                             |
|------------------------|--------------------|---------------------------------------------------------|-----------------------------|
|                        |                    | Spindle elongation                                      | Chromosome to pole movement |
| 1.25 mM ADP            | 10                 | $0.04 \pm 0.08$                                         | $0.15 \pm 0.06^\ddagger$    |
| 1.25 mM ATP            | 8                  | $0.30 \pm 0.13^{*\ddagger}$                             | $0.13 \pm 0.04$             |
| 5 mM C~P               | 10                 | $0.09 \pm 0.07$                                         | $0.13 \pm 0.04$             |
| 0.5 mM ADP + 5 mM C~P  | 6                  | $0.13 \pm 0.08^*$                                       | $0.18 \pm 0.06$             |
| 1.25 mM ADP + 5 mM C~P | 8                  | $0.18 \pm 0.09^{*\ddagger}$                             | $0.15 \pm 0.06$             |
| 3 mM ADP + 5 mM C~P    | 6                  | $0.25 \pm 0.14^{*\ddagger}$                             | $0.16 \pm 0.07$             |

Rates of movement were estimated by measuring the slopes of graphs derived from the cine records of chromosome–chromosome and pole–pole distances from the moment of addition of step 2 medium to the position of maximum displacement of chromosomes or poles. The rate of chromosome to pole movement was calculated as one-half the difference between the rates of chromosome separation and spindle elongation<sup>8,10</sup>. Values are given with standard deviation. C~P, phosphoryl creatine.

\* The differences between means (versus 1.25 mM ADP) were statistically significant at  $P < 0.02$  using Student's *t*-test.

† The differences between means (versus 5 mM C~P) were statistically significant at  $P < 0.05$  using Student's *t*-test.

‡ In this column the differences between means (versus 1.25 mM ADP) were not statistically significant using Student's *t*-test.

Cells lysed in either ADP or phosphoryl creatine alone display little or no spindle elongation (Fig. 1a,b, Table 1); however, cells lysed in ADP and phosphoryl creatine display extensive spindle elongation at approximately two-thirds the rate observed in an ATP concentration equivalent to the ADP level present (Fig. 1c, Table 1). I have also studied the ADP dependence of anaphase B with a constant level of phosphoryl creatine present in the lysis medium (Table 1). In these experiments the mean rate of spindle elongation is dependent on the concentration of ADP in the lysis medium, and the concentration range over which this dependence is demonstrated is similar to that described previously for ATP alone<sup>8</sup>.

The results described here are consistent with the observation made by indirect immunofluorescence that creatine kinase is bound to a fibrous spindle component that is distributed both in the spindle midzone and near the spindle poles<sup>4</sup>. I can explain my results only if endogenously retained creatine kinase in the presence of ADP and phosphoryl creatine in the lysis medium provides the ATP required for spindle elongation.

These results support previous demonstrations that anaphase A and anaphase B are physiologically distinct processes<sup>8,9,11,12</sup> because only anaphase B is affected by addition of ADP and phosphoryl creatine to the lysis medium. As Koons *et al.*<sup>4</sup> have demonstrated that the creatine kinase inhibitor epoxycrystallin blocks cell division by retarding centriole (and spindle pole) separation during prophase, these results also suggest that spindle pole separation during anaphase and prophase may be closely related processes involving a dynein-like ATPase and creatine kinase as an ATP regenerating system.

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- Carlson, F. D. & Siger, A. *J. gen. Physiol.* **44**, 33–60 (1960).
- Cain, D. F. & Davies, R. E. *Biochem. biophys. Res. Commun.* **8**, 361–366 (1962).
- Eckert, B. S., Koons, S. J., Schantz, A. W. & Zobel, C. R. *J. Cell Biol.* **86**, 1–5 (1980).
- Koons, S. J., Eckert, B. S. & Zobel, C. R. *Expl Cell Res.* **140**, 401–409 (1982).
- Fuseler, J. W., Eckert, B. S., Koons, S. J. & Shay, J. W. *Cell and Muscle Motility* Vol. 2 (eds Dowben, R. M. & Shay, J. W.) 103–119 (1982).
- Silver, R. B., Saft, M. S., Taylor, D. A. & Cole, R. D. *J. Cell Biol.* **95**, abstr., 306 (1982).
- Erashova, S., Saks, V. A., Sharov, V. G. & Lyzova, S. N. *Biokhimiya* **44**, 295–299 (1979).
- Cande, W. Z. *Cell* **28**, 15–22 (1982).
- Cande, W. Z. *Nature* **295**, 700–701 (1982).
- Cande, W. Z. *Meth. Cell Biol.* **25B**, 57–68 (1982).
- Ris, H. *Biol. Bull.* **96**, 90–106 (1949).
- Pickett-Heaps, J. D., Tippit, D. H. & Porter, K. R. *Cell* **29**, 729–744 (1982).



## Determination of the absolute handedness of knots and catenanes of DNA

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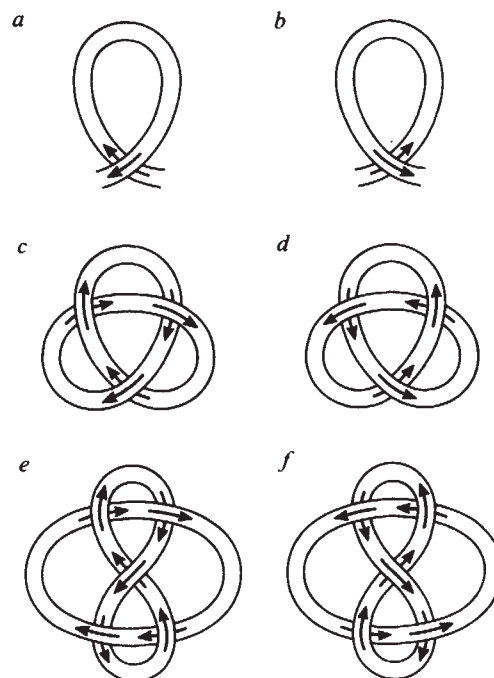
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DNA winds about itself in a right-handed or left-handed fashion at several structural levels. The double helix is generally right-handed and is given a (+) sign by convention, whereas supercoiling of the helix axis is always (−) in the cell<sup>1,2</sup>. The winding in higher order forms such as knots and catenanes is unknown, and this has impeded elucidation of the mechanisms of their formation and resolution by replication, recombination and topoisomerase action<sup>3–6</sup>. We introduce here a procedure for determining the handedness of DNA winding by inspection of electron micrographs of DNA molecules coated with *Escherichia coli* RecA protein. We demonstrate the validity of the method and show that DNA topoisomerase I of *E. coli*<sup>7</sup> generates an equal mixture of (+) and (−) duplex DNA knots, and that one product of recombination by resolvase of transposon Tn3 (refs 8, 9) is a catenane of uniquely (+) sign.

We have adopted a sign convention that can be applied uniformly to the winding in catenanes, knots, supercoils and double helices. The plane projection of two crossing DNA segments can form a node in two topologically distinct ways (for supercoils, see Fig. 1a, b). The crossing in a left-handed supercoil is given a (−) sign and that in a right-handed supercoil a (+) sign. The arrows show the relative direction of the two segments along the DNA backbone. To determine the sign of any node, both the relative direction and the overlay of the crossing DNA segments must be distinguished.

It is straightforward to determine the direction of crossing segments in intramolecular nodes in supercoiled or knotted rings, because the path of DNA connecting the segments can be followed. To describe the sign of a knot then, it is only necessary to determine reliably which of the crossing segments overlies the other. This has not been possible by electron microscopic examination of conventionally stained and shadowed DNA because resolution of the two segments at the junction is poor, with rare exceptions<sup>17</sup>. To overcome this problem, we sought a protein coat for the DNA that would enhance visualization of the nodes; *E. coli* RecA protein<sup>10,11</sup> proved ideal. In the presence of ATPγS, it binds cooperatively to duplex DNA to form a stiffened striated complex about 100 Å in diameter<sup>12</sup>. Figure 2 shows RecA-decorated trefoil knots that have been tied in nicked rings by *E. coli* DNA topoisomerase I. The three nodes are (+) in the upper knot (Fig. 2a) and (−) in the lower knot (Fig. 2b).

To validate our procedure for determining the chirality of knots, we took advantage of the topological requirement that trefoils have either three (−) (Fig. 1c) or three (+) (Fig. 1d) nodes. Thus, every knot provided two internal checks on the accuracy of each node assignment. A mask was placed over a micrograph so that only a single node of a knot was visible at a time. Three observers independently scored which of the crossing segments was on top. Ninety-two per cent of the nodes in the 47 trefoils analysed were assigned the same sign as the other two nodes of the knot. By considering exclusively knots

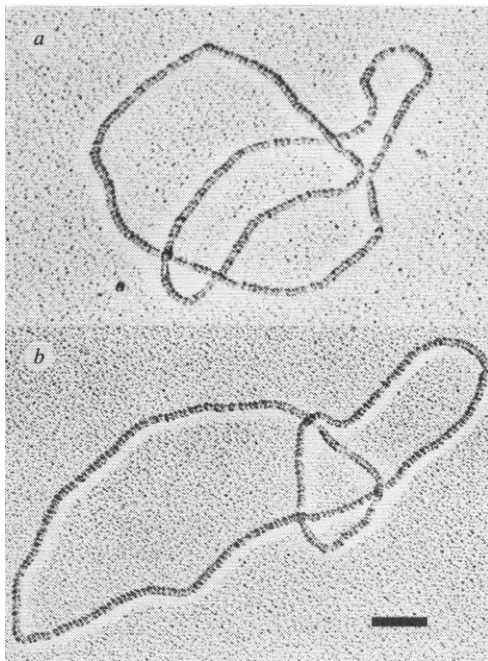


**Fig. 1** Topology of nodes, trefoil knots and figure-8 catenanes. Sign of nodes: as illustrated for supercoils, two DNA segments can cross in plane projection to form nodes of two topological types: (−) in a and (+) in b. The arrows indicate the path of the connecting DNA. Node sign is a local property dictated only by the overlay and relative direction of the crossing segments. The absolute direction of the arrows is arbitrary because traversing the path in the opposite direction maintains the sign. Trefoil knots: these can have either three (−) nodes (c) or three (+) nodes (d). Figure-8 catenanes: four of the nodes of figure-8 catenanes are intermolecular: two are (+) and two are (−). The central node describes a supercoil entrapped by the intermolecular linkage and thereby defines the two stereoisomers of figure-8 catenanes. The (−) catenane (e) contains a left-handed supercoil and the (+) catenane (f) a right-handed supercoil.

where the consensus sign for all three nodes was the same, the probability of incorrectly ascribing sign to a knot is negligible.

Of the 40 knots generated by topoisomerase I that met this restriction, 18 were (+) and 22 were (−). The difference of this ratio from 1 is not statistically significant. In knotting DNA rings, DNA topoisomerase I passes a duplex DNA segment through the intact strand remaining at a nick<sup>13</sup>, thereby changing the sign of the crossed segments. As topoisomerase I produced (+) and (−) knots equally, (+) and (−) nodes must be formed and inverted equally in its active centre. Previously topoisomerase I had been shown to invert (−) nodes of only one sign, because it relaxed (−), but not (+), supercoils<sup>7</sup>. This substantiates the view of topoisomerases as enzymes that can promote permeation of DNA segments through each other without regard to sign or orientation of the crossing segments.

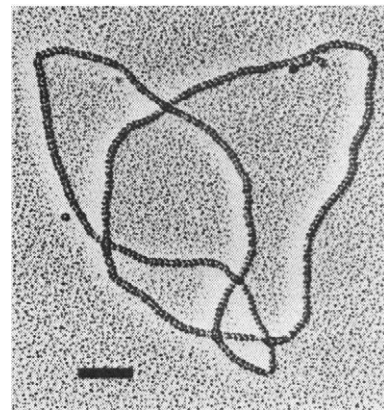
By contrast with the racemic topoisomerase I products, the site-specific recombination enzyme Tn3 resolvase generated a unique stereoisomer. This enzyme promotes recombination between directly repeated resolution sites in a supercoiled plasmid to give two linked daughter rings<sup>8,9</sup>. About 99% of the recombinants are singly interlinked catenanes<sup>9</sup>. When the products were nicked to remove supercoils, a minor fraction was revealed that had a higher electrophoretic mobility. We expected that these would be conventional doubly-interlinked catenanes but found instead that they have the novel topological form of a figure-8 catenane. The two possible stereoisomers of figure-8 catenanes (Fig. 1e, f), can be distinguished simply by inspection of the path and overlay of the DNA. In contrast to standard catenanes, the relative direction of the two component rings need not be distinguished. In the simplest representation of a figure-8 catenane (Fig. 1e, f), there are two (+) and two



**Fig. 2** Electron micrographs of RecA protein-coated trefoil knots generated by *E. coli* DNA topoisomerase I. Forty microgrammes of plasmid pRR51 (ref. 8) were singly nicked by pancreatic DNase I in the presence of ethidium bromide and knotted by treatment with 60  $\mu$ g of *E. coli* topoisomerase I in a 2.4-ml reaction mixture containing 30% (v/v) glycerol, 20 mM Tris-HCl (pH 7.6), 20 mM KCl, 8 mM potassium phosphate, 6 mM MgCl<sub>2</sub> and 0.5 mM dithiothreitol. After 30 min at 52 °C, the products were separated by electrophoresis through a 0.8% agarose gel and the trefoil knots (about 10% of the product) isolated by electroelution. Twenty-five nanogrammes of the purified DNA were incubated at 37 °C with 1  $\mu$ g of RecA protein<sup>15</sup> in a 20- $\mu$ l reaction mixture containing 25 mM triethanolamine chloride (pH 7.6), 2.5 mM magnesium acetate and 2 mM rATP. After 5 min, 0.5 mM ATP $\gamma$ S was added to stabilize the complexes, and 30 min later glutaraldehyde was added to 0.2%. After an additional 15 min incubation, the reaction mixture was applied to a 0.5 ml Sepharose 2B (Pharmacia) column and eluted with 5 mM magnesium acetate. The drops were collected on Parafilm and adsorbed to a freshly glow discharged carbon-coated grid for electron microscopy<sup>16</sup>. The specimens were washed by floating on 5 mM magnesium acetate for 20 min, dehydrated in absolute ethanol and rotary shadowed at an angle of 7° with carbon-platinum. The striations on the DNA correspond to the right-handed helical coating by RecA<sup>12</sup> and provide an internal control for the correct reading of the photographic negatives. Scale bar, 1,000 Å.

(-) intermolecular nodes that entrap a supercoil in one of the component rings. Because the signs of the intermolecular nodes cancel, the sign of the whole molecule is that of the lone intramolecular node. The catenane with the left-handed supercoil (Fig. 1e) is (-), and the one with the right-handed supercoil (Fig. 1f) is (+). We determined the sign of the resolvase product as above by masking the DNA so that only one of the five nodes was visible at a time and scoring the overlying DNA segment. For the 16 molecules analysed by two observers, 93% of the node signs were consistent. After eliminating three molecules whose structure was ambiguous, the consistency was 98%. All 13 of the remaining figure-8 catenanes were (+) (see Fig. 3).

Three features of the recombination mechanism are implied by this single result, barring the unlikely possibility that (-) catenanes were also produced but selectively lost. First, resolvase pairs the recombination sites and rearranges the broken DNA strands in a well defined fashion to generate a product with unique handedness. Second, if an achiral DNA intermediate were generated in recombination, then resolvase must form an asymmetric complex with the intermediate<sup>14</sup>. Third, the



**Fig. 3** Electron micrograph of a figure-8 catenane produced by Tn3 resolvase. 225  $\mu$ g of plasmid p51A<sup>2</sup>, containing directly repeated copies of the recombination site, were treated with resolvase and nicked with DNase I in the presence of ethidium bromide as described earlier<sup>9</sup>. The products were separated by agarose gel electrophoresis and about 1% were figure-8 catenanes which migrated faster than the predominant product of singly interlinked daughter rings. The figure-8 catenanes were isolated, coated with RecA protein and prepared for electron microscopy as described in Fig. 2 legend. Scale bar, 1,000 Å.

catenated product of recombination contains an entrapped (+) supercoil, but the substrate is negatively supercoiled. Thus, catenation here can hardly result from a passive redistribution of supercoil windings as has been suggested for another recombination system<sup>4</sup>, but instead must be directed by the enzyme.

All mechanisms for generation of knots and catenanes make implicit predictions of sign, and these can now be tested directly. Conversely, the sign of naturally occurring knots and catenanes will illuminate the history of their formation since we have shown that enzymes leave a characteristic topological fingerprint. Such structural studies are greatly facilitated by the DNA visualization technique used here because it allows unambiguous identification of complex topological forms by distinguishing nodes resulting from true linkage from accidental foldovers.

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1. Crick, F. H. C., Wang, J. C. & Bauer, W. R. *J. molec. Biol.* **129**, 449-461 (1979).
2. Dickerson, R. E. *et al. Science* **216**, 475-485 (1982).
3. Sundin, O. & Varshavsky, A. *Cell* **21**, 103-114 (1980).
4. Mizuuchi, K., Gellert, M., Weisberg, R. A. & Nash, H. A. *J. molec. Biol.* **141**, 485-494 (1980).
5. Liu, L. F., Liu, C. C. & Alberts, B. M. *Cell* **19**, 697-707 (1980).
6. Kreuzer, K. N. & Cozzarelli, N. R. *Cell* **20**, 245-254 (1980).
7. Wang, J. C. & Liu, L. F. in *Molecular Genetics Pt III* (ed. Taylor, J. H.) 65-88 (Academic, New York, 1979).
8. Reed, R. R. *Cell* **25**, 713-719 (1981).
9. Krasnow, M. A. & Cozzarelli, N. R. *Cell* **32**, 1313-1324 (1983).
10. Radding, C. M. *Cell* **25**, 3-4 (1981).
11. McEntee, K. & Weinstock, G. M. *The Enzymes* **14a**, 445-470 (1981).
12. Di Capua, E., Engel, A., Stasiak, A. & Koller, Th. *J. molec. Biol.* **157**, 87-103 (1982).
13. Dean, F. *et al. Cold Spring Harb. Symp. quant. Biol.* **47**, 769-777 (1983).
14. Ogston, A. G. *Nature* **162**, 963 (1948).
15. Stasiak, A. & Di Capua, E. *Nature* **299**, 185-186 (1982).
16. Arcidiacono, A., Stasiak, A. & Koller, Th. *Proc. 7th Eur. Congr. on Electron Microscopy* **2**, 516-523 (1980).
17. Hudson, B. & Vinograd, J. *Nature* **216**, 647-652 (1967).

## Erratum

In the article 'Embryonic lethal mutation in mice induced by retrovirus insertion into the  $\alpha 1(I)$  collagen gene' by Angelika Schnieke, Klaus Harbers and Rudolf Jaenisch, *Nature* **304**, 315-320 (1983), on page 317, Figure 3B and Figure 2B were transposed.



## Crustal thinning and subsidence in the North Sea

WOOD AND BARTON<sup>1</sup> based their analysis of the North Sea Rift on gravity, refraction and well data but did not have access to multichannel reflection data. Their conclusion that the North Sea Rift subsided in response to an extension of the crust by 110 km, 50–80 km of which occurred during the mid-Jurassic–early Cretaceous, is at variance with reflection seismic data acquired by the petroleum industry which indicate an extension of only 20–30 km at the base Zechstein Salt level.

In the central North Sea, the Zechstein Salt forms part of the pre-rift sequence and its base is a regionally correlative stratigraphical and seismic marker. Thus the extension mapped at this level gives an upper limit to the amount of extension that can have occurred during the Mesozoic rifting stage of the North Sea. Amounts of extension by faulting at the base Zechstein Salt level are probably 20–25 km, but could be as large as 30 km. It is very unlikely, however, that a multitude of additional faults, undetectable on multichannel seismic reflection data, could account for the doubling or quadrupling of this amount as required by the stretching model<sup>2</sup>.

Average stretching factors for the central North Sea area derived from both refraction and reflection data are as follows:

|                     | Crustal configuration | Base Zechstein reflector |
|---------------------|-----------------------|--------------------------|
| Total cross-section | 1.25                  | 1.04–1.06                |
| Central Graben only | 2.00                  | 1.1–1.15                 |

The serious discrepancies between these stretching factors suggest that crustal attenuation during the Mesozoic rifting stage of the North Sea Rift was not achieved solely by mechanical extension of the crust but that other rift-related mechanisms have also contributed to crustal thinning. Such a mechanism could be 'sub-crustal erosion' involving progressive intrusion of mantle material into the lower crust (dykes, sills) and its gradual assimilation into the upper mantle, thus causing a permanent upward displacement of the crust–mantle boundary. Such processes were probably most active during the mid-Jurassic doming stage of the central North Sea area.

Doming of rifts can be related to the ascent of the asthenosphere–lithosphere boundary (mantle plume model) and/or to the diapiric ascent of hot asthenospheric material to the base of the crust where it spreads out laterally (tensional failure model<sup>3,4</sup>). This latter process is the more likely mechanism to have caused the short-lived mid-Jurassic uplift of the central North Sea area. Emplacement of such

an asthenolith and its subsequent cooling and resorption into the upper mantle, combined with further crustal extension, can explain the mid-Jurassic–Cretaceous subsidence pattern of the central North Sea area.

The thickness of the Cenozoic strata indicates that the thermal anomaly governing the post-rifting subsidence of the North Sea Rift was largest in the area that was domed-up during the mid-Jurassic. On the basis of subsidence analyses, Wood and Barton postulate a  $\beta$ -value of 1.5 for the Central Graben while reflection data indicate a  $\beta$ -value of 1.1–1.15. This discrepancy suggests that the magnitude of a thermal anomaly derived from the post-rifting subsidence of a rift cannot be readily related to a stretching factor. Similar discrepancies between stretching factors derived from subsidence analyses, crustal configuration and reflection data are also observed in the Witch Ground–Buchan grabens<sup>5,6</sup> and the Viking Graben of the North Sea<sup>7,8</sup>.

The crustal configuration of the central North Sea, as defined by the available gravity and refraction data, combined with the  $\beta$ -value derived from the geometry of the pre-rift sediments, suggests that syn-rift crustal attenuation was achieved not only by crustal stretching but also to a large extent by 'sub-crustal erosion'. Additional geophysical data, such as deep crustal reflection profiles, are required to clarify these issues.

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1. Wood, R. & Barton, P. *Nature* **302**, 134–136 (1983).
2. McKenzie, D. P. *Earth planet. Sci. Lett.* **40**, 25–32 (1978).
3. Turcotte, D. L. *Lunar planet. Inst. Contr.* **451**, 5–8 (1981).
4. Neugebauer, H. W. et al. *Plateau Uplift, the Rhenish Massif, a Case History* (Springer, Berlin, in the press).
5. Christie, P. A. & Sclater, J. G. *Nature* **283**, 729–732 (1980).
6. Smythe, D. K., Skuce, A. G. & Donato, J. A. *Nature* **287**, 467–468 (1980).
7. Ziegler, P. A. *Geological Atlas of Western and Central Europe* (Elsevier, Amsterdam, 1982).
8. Sclater, J. G. & Christie, P. A. *J. geophys. Res.* **85**, 3711–3730 (1980).

BARTON AND WOOD REPLY—Does Ziegler speak for the whole petroleum industry on this matter? Our correspondence with oil companies suggests that different estimates of extension are made from similar multichannel seismic reflection data. An example of these discrepancies is seen in the interpretation of tilted fault block structures in northern Biscay, where estimates of extension vary between 15%<sup>1</sup> and 200–300%<sup>2</sup>. There are three possible explanations for these discrepancies:

(1) Seismic resolution of the deep basement and its faults improves with each

advance in data acquisition and processing. As previously hidden faults are exposed, estimates of  $\beta$  will increase.

(2) The amount of overall extension represented by the configuration of the visible part of a fault is highly controversial<sup>3,4</sup>. The reconstruction depends on the geometric interpretation of the fault<sup>1,2</sup>, as well as the velocities used in the time–depth conversion<sup>3</sup>.

(3) The visible high-angle fault breaks may represent only the most recent generation of faulting affecting the basin. In the Basin and Range province of the United States<sup>5</sup> and the Aegean Sea (J. A. Jackson, personal communication), earlier generations of faults, now rotated to flat lying, have taken up much of the extension. Such faults may be seismically obscure, and may also exist in the North Sea.

Any serious departure between estimates of  $\beta$  from reflection methods, as opposed to refraction and subsidence studies, which still remained after the resolution of the above three points would become a real problem. If, in that case, a secondary mechanism of thinning were indicated, we would be reluctant to invoke an *ad hoc* process such as 'sub-crustal erosion'; a speculative concept with no quantitative basis for testing.

Western Geophysical have recently acquired two short deep reflection profiles for BIRPS (British Institutions Reflection Profiling Syndicate) along the refraction line and these are being examined.

Cambridge Earth Science contribution no. 386.

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1. Montadert, L., Roberts, D. G., De Charpal, O. & Guenoc, P. *Init. Rep. DSDP Leg 48*, 1025–1060 (1979).
2. Le Pichon, X., & Sibuet, J.-C. *J. geophys. Res.* **86**, (B5), 3708–3720 (1981).
3. Foucher, J.-P., Le Pichon, X. & Sibuet, J.-C. *Phil. Trans. R. Soc. A* **305**, 27–43 (1982).
4. Bally, A. W. *Phil. Trans. R. Soc. A* **305**, 325–336 (1982).
5. Proffett, J. M. *Bull. geol. Soc. Am.* **88**, 247–266 (1977).

## A transcriptional function for repetitive ribosomal spacers in *Xenopus*?

MOSS<sup>1</sup> has recently identified and examined *in vivo* transcripts of the *Xenopus laevis* nontranscribed spacer by northern blot and S<sub>1</sub> nuclease analyses. He has proposed that "the ribosomal spacer is a loading site for RNA polymerase I, and spacer transcription is the driving force by which polymerase is delivered to the ribosomal gene pro-

moter". Here we present considerations that tend to invalidate Moss' interpretation of his data as a general model.

The major flaw in Moss' model is that it is based on a characterization of spacer transcription that disregards quantitative analysis of *in vivo* transcripts. Specifically, spacer transcripts are too rare to be a component of normal ribosomal DNA (rDNA) expression. This has been apparent in electron microscopic analyses of spacer activity in *Xenopus* oocytes, where short ribonucleoprotein matrices ('prelude transcripts') are infrequently observed in the rDNA spacer<sup>2</sup>. Following identification of the site of initiation of *Xenopus* rRNA precursor synthesis<sup>3</sup>, and the observation of homology between repeated spacer elements (the so-called *Bam* islands) and the initiation region<sup>4</sup>, Trendelenburg<sup>5</sup> reexamined spacer transcripts in detail. One of his findings was that, while prelude transcripts could be found in 50–70% of the spacers of a few frogs (3/25), in most frogs, only 2–5% of rDNA spacers had transcription matrices, and yet other frogs apparently had none. Thus, in the most favourable of examples from individual frogs, only two-thirds of the spacers showed prelude transcription, and in a randomly selected frog, the frequency was  $\leq 1/20$  this fraction. Such frequencies indicate that spacer transcription is generally rare in *Xenopus*, and, therefore, unlikely to be "the driving force by which polymerase is delivered to the ribosomal gene promoter".

The scarcity of spacer transcripts is also shown by biochemical estimates of their abundance in both *Xenopus* and *Drosophila*. Sollner-Webb and Schultz (cited in ref. 6) have found that in RNA of different frogs, the amount of 5' end of spacer transcripts relative to the amount of 5' end of 40S RNA varies from ~1% to <0.05%. This is in good agreement with the electron microscopic observations. In *Drosophila*, a variable number of copies of initiation sequences are tandemly repeated immediately upstream of the rRNA transcription unit<sup>7</sup>. In both *Drosophila melanogaster* and *Drosophila virilis*, we have found short nuclear RNAs that result from transcription of these NTS repeats. The transcripts are rare, their steady-state level being ~1% that of the steady-state level of the external transcribed spacer portion of the ribosomal RNA precursor. These quantitative points argue against a major role of spacer transcription in the expression of rRNA genes.

Although transcriptional activity of the NTS repeats is rare, the possibility remains that multiple copies of promoter sequences in some way facilitate rRNA precursor initiation. However, the duplication of promoter sequences seen in the spacers of *Xenopus*, *Drosophila* and some other organisms is not a ubiquitous feature of rRNA genes, as such duplications are absent from the rDNA of yeast<sup>8</sup>

and humans<sup>9</sup>. Therefore, the existence of multiple sites for potential transcription falls short as a general model. An alternative explanation is that the spacer repeats of *Xenopus* and *Drosophila* have been generated through unequal crossing-over at points of adventitious sequence homology during the course of evolution, but serve no function in a regulatory capacity. As the repeats contain signals for transcription initiation, accidental transcripts may be generated. Present evidence neither supports nor eliminates either of these models, and assignment of any function to the spacer transcripts is premature.

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1. Moss, T. *Nature* **302**, 233–238 (1983).
2. Scheer, U., Trendelenburg, M. F. & Franke, W. W. *Expl Cell Res.* **80**, 175–190 (1973).
3. Sollner-Webb, B. & Reeder, R. H. *Cell* **18**, 485–499 (1979).
4. Boseley, P. G., Moss, T., Machler, M., Portmann, R. & Birnstiel, M. L. *Cell* **17**, 19–31 (1979).
5. Trendelenburg, M. F. *Biol. Cell* **42**, 1–12 (1981).
6. Sollner-Webb, B., Wilkinson, J. K. & Miller, K. G. *The Cell Nucleus* Vol. 12, 31–67 (Academic, New York, 1982).
7. Kohorn, B. D. & Rae, P. M. M. *Nucleic Acids Res.* **10**, 6879–6886 (1982).
8. Valenzuela, P. et al. *J. biol. Chem.* **252**, 8126–8135 (1977).
9. Miesfeld, R. & Arnheim, N. *Nucleic Acids Res.* **10**, 3933–3949 (1982).

**MOSS REPLIES**—The criticisms of Murtif and Rae stem mainly from a misunderstanding of the details of the *Xenopus laevis* ribosomal spacer model<sup>1</sup>. The model proposed for the function of this spacer was developed from biochemical observations of spacer transcription *in vivo*<sup>1</sup> and from earlier studies of the primary structure of this spacer<sup>2</sup>. However, spacer promotion alone is insufficient to explain the strong competition observed between spacer-containing and spacer-less genes<sup>1</sup>. It was therefore postulated<sup>1</sup> that the highly repetitive spacer sequences were also active in this competition. As these sequences would be unable to initiate spacer transcription but show some promoter homologies, it was suggested that they may act as RNA polymerase binding sites. Hence in the model a certain level of transcription, initiated at the spacer promoters, acts to drive bound, but uninitiated, polymerase to the major (40S) gene promoter. Each spacer need direct only one transcript at a time for its optimal functioning by this mechanism. Therefore, the number of spacer transcripts produced at maximal gene activity could be as low as 5% of the 40S RNA transcription (the ratio of spacer promoters to repetitive polymerase binding sites) or even 2.5% if close-packing of uninitiated polymerase were possible. Sollner-Webb and Schultz (cited in ref. 3) estimate the number of 5' termini of spacer transcripts in *X. laevis* tissue as

between 0.05 and 1% of that of 40S RNA 5' termini at steady state. I have observed very similar levels in *X. laevis* tissue culture (ref. 1 and my unpublished observations). However, I have also observed in the same RNA preparations that the number of 3' termini of these spacer transcripts is ~10% of 40S 5' termini. Therefore the 5' and 3' termini of the spacer transcripts have very different half lives within the cell. Thus, Sollner-Webb and Schultz probably greatly underestimate the steady-state level of these transcripts.

It is, of course, not the steady-state RNA levels, but the relative rates of 40S RNA and spacer RNA initiation which are relevant to tests of the model of spacer function. My preliminary results indicate that compared with the 40S RNA, spacer transcripts are more highly represented in pulse-labelled RNA than in steady-state RNA. They are therefore more rapidly degraded than the 40S RNA and any measurements made on steady-state RNA levels will underestimate the true rate of spacer transcription.

Data on ribosomal spacer transcription obtained from electron microscope analyses are by necessity rather selective. Additionally, electron microscope analyses are subject to the same problems as are the steady-state biochemical analyses if, as is likely, the half life of the spacer transcripts is about the same as, or less than, the sample preparation time. The work of Rungger *et al.*<sup>4</sup> demonstrates that agents assumed to slow RNA processing or degradation also increase the spacer transcription detected in electron microscope spreads. Thus, spacer transcripts may be degraded during normal spreading. Nevertheless, Scheer *et al.*<sup>5</sup> studying *Triturus*, not *Xenopus*, say of their observations "Frequently ... lateral fibrils (transcripts) are seen attached to the spacer segments ...".

It was not suggested in ref. 1 that the proposed model is generally applicable, nor should one necessarily expect it to be so. If the model is appropriate to other *Xenopus* species and *Drosophila melanogaster*, this will be deduced from functional assays. The usefulness of the model in *X. laevis* is not reduced by its apparent inapplicability to yeast and human ribosomal spacers, neither of which have been completely sequenced.

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2. Moss, T., Boseley, P. G. & Birnstiel, M. L. *Nucleic Acids Res.* **6**, 3733–3743 (1979).
3. Sollner-Webb, B., Wilkinson, J. K. & Miller, K. G. *The Cell Nucleus* Vol. 12, 31–67 (Academic, New York, 1982).
4. Rungger, D., Crippa, M., Trendelenburg, M. F., Scheer, U. & Franke, W. W. *Expl Cell Res.* **116**, 481–486 (1978).
5. Scheer, U., Trendelenburg, M. F. & Franke, W. W. *Expl Cell Res.* **80**, 175–190 (1973).



# Methods in March-hare madness

W.D. Hamilton

**Mate Choice.** Edited by Patrick Bateson.

Cambridge University Press: 1983. Pp.460. Hbk £30, \$59.50; pbk £12.50, \$19.95.

SENATOR Proxmire, scourge of "useless" science, has been quoted as saying that high on his list of topics that the public does not need to have researched is why people fall in love. Proxmire notwithstanding, insatiable scientists are trying hard to find answers to the question. Currently there is an effort greater than ever before to understand both sexuality itself and all manifestations that result from it. *Mate Choice* is one of the most recent expressions of that effort.

The book is based on a symposium held in England in 1981. The editor, Patrick Bateson, is a distinguished contributor to the field and the collection of essays brings together much of the recent work and ideas. The book is not, it should be said at once, about human mate choice. Rather it is about animals, and, reflecting both the editor's own interest and most current research, birds are covered in by far the most detail. Amphibia, rodents, rabbits and primates all receive chapters, (but no invertebrate group has one). One chapter specifically addresses man, but this is not particularly useful since it lacks the broad evolutionary perspective that unites most of the other contributions.

Despite the absence, by and large, of man, the book certainly cannot be read without thought of him (and her). Or indeed without noticing the busy trade in terms and ideas between, on the one hand, the exciting, agonizing world we all know so well from the inside and, on the other, the parody world of the animals that we can observe a little more objectively — a world of nest and plume and song and tiny combat. How seriously one takes the correspondence to human behaviour depends greatly on temperament, but it can be said that the more animals are studied the more human their problems and reactions seem. There is even some return to a pre-ethological, perhaps pre-scientific, acceptance of a trade in comparisons without limit. The term "cuckold" for example, mediaevally borrowed from folk observation of the nest-usurping habits of the female cuckoo and her offspring, has now come back into the world of animal study with its special human meaning: soon we may learn that, like men, male cuckoos behave as if they were worried about cuckoldry, though there is little of this in the book.

For the most part the data-orientated parts are well representative of what is now known, at least about vertebrates. Among those chapters which seem to me particularly valuable are the review of re-

mating in birds by Rowley (an inexhaustible contributor of new facts) and a more general review by Wickler and Seibt on monogamy. Among the more specialized contributions, I particularly appreciated the summaries of long-term studies of pairing of kittiwakes by Coulson, and of snow geese by Cooke and Davies. As these chapters help to remind one, the diversity of mating habits of birds brackets us on all sides. Some cases of monogamy in birds almost outmatch even the highest human ideals of fidelity, attachments in birds lasting sometimes even after death (Wickler and Seibt). But even monogamous birds are not perfect either: there are hints of this in the book and the missing chapter on monogamy could have shown more. Away at the other extreme there are those cases of polygyny gone wild where one male may father almost all of a local population.

What have theorists been doing while the new surge of data rolls in? Three interlinked themes, well represented in the book, may be mentioned briefly. First, is there any *real* female choice? Second, granted choice, does R.A. Fisher's "runaway" process of sexual selection really run; and does it underlie the exaggerated and the ornate in animal display to the extent that he supposed? Third, whether or not there exist genes subject to the largely *arbitrary* preferences addressed in the last question, are females looking also for *utilitarian* "good genes" for their offspring — genes concerned with non-sexual challenges in the battle for life?

Real female preference is now known for many species and is well documented in several chapters. Some authors, however (Halliday for example), express doubt that it has much significance. More usually females simply accept the winners of competitions. They may hardly look at the male's characteristics directly, seeming only to care that he has triumphed over his rivals. But even victoriousness often seems not to be the real issue; females may merely be selecting the food or the real estate that he has come to control. If all were merely a matter of choice for the best resources for the brood (obviously a large factor in many cases), there would be no great puzzle — but at the same time there would be no element of Darwinian sexual selection, as Arnold's chapter points out.

Close inspection, however, often reveals that there is actually something more. A female could easily avoid the hectic commotion of a lek (mating gathering) and mate outside if she so chose. Or, in a territorial species, she could simply go for some male of middling quality: she could then have his little-contested but still far from worthless territory all to herself, instead of the mere portion of a good territory she actually accepts when she goes with other females to the strong male at (say) the centre of the marsh. On the evidence of females picking less-than-best resources, or ignoring resources altogether as in the lek, it seems that "good genes" must often be one factor in their choice.

Then what are they after? If, and only if, females choose their mates by personal sensory inspection, then R.A. Fisher's "runaway" process might be the answer. In this process coevolving female preference is supposed to become genetically correlated with, and so escalate, the selection of male characteristics. Fisher thought the male characteristic should have an initial advantage to get it started, but one recent theorist (Kirkpatrick) finds the escalation effect to be so powerful that random fluctuation of frequency can initiate the process. To caricature this recent view, it is as if a buxom hitch-hiker called Preference conjures up the car that is to carry her out of an old tyre that she kicks into the road. (Later, as Kirkpatrick implied, she would be apt to smash all into the ditch again, herself included.)

If you want to know more about this process or non-process, whichever it is, then read this book; here you may choose from an almost repetitious selection of accounts. In a lucid chapter marred by a tendency to suppose that all that is worth saying about sexual selection has been said a decade or more ago, O'Donald concludes that the "runaway" doesn't really work; Arnold, in an equally lucid chapter marred by a tendency to wave aside "good genes" other than those of the sexy Fisher-Lande type, supports Lande's claim that in an extended and hopefully more realistic poly-

IMAGE  
UNAVAILABLE  
FOR  
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REASONS

*Male peacock displays its tail — to what effect?*

genic model Fisher's heuristic vision is upheld.

In my own view the bizarre, the exaggerated, the seemingly arbitrary in animal ornament speaks strongly for the idea of positive feedback processes occurring at least sporadically, but the point that such feedback can't occur if fighting rules and real choice is absent shows the intra-sex competition must be able to produce highly bizarre weapons and displays on its own. In other words, facts suggest what theory can confirm — it pays to advertise, by some striking possession, your strength if you have it. Otherwise you may waste energy and even gore in proving your superiority to lesser rivals. This point is not well brought out in any of the papers, even though it helps in explaining much that might otherwise suggest the "run-away" selection process. A further point is that to look for winners of combats is exactly what females *should* do if they sought for their species a moderate alternative to the dangerous dysgenics of the Fisher, Lande and Kirkpatrick process. Perhaps we tend to see around us mainly the species that took this route and survived — well bred and forewarned, so to speak, they are species that on seeing deadly Preference on the road hasten by on the other side.

Now to the third question. Why should the female (as it usually is), the hitch-hiker yet unthought of, ever *start* seeking anything like all this? Here, most of the authors are rather against the possibility of a large supply of unfixed good genes of utilitarian purpose. Some, however, for example Arnold, readily pre-suppose an undiminishing supply of the Fisher-Lande character genes, arguing like Lande that this supply can be as constantly topped up by mutation as it is depleted by the sexual selection. A few authors invoke a similar mutational maintenance of variance for utilitarian characters. But in my opinion this support is still inadequate to the case. For the whole crazy sexual carnival to make sense, a large and essentially undepletable utilitarian variance needs to be there. Such variance, I believe, may be reasonably derived from genetic disease and parasite resistance genes that are kept moving in slow dynamic polymorphism by Lotka-Volterra-type frequency-dependent processes.

In 1975, Zahavi also suggested a persistent utilitarian function for sexual selection and tied it in with a seemingly perverse notion that selectors are looking for physical handicaps that prove the potential mate's vigour. This idea gets short shrift in the book. Only three of the more theoretical writers even throw a nod towards it, all of them quizzically at that. Although it was carelessly presented, I believe that Zahavi's general notion is sustainable. Attacks on the letter of what he said were justified, but sympathy to a good half of the idea would have been justified too. Dominey has already neatly

shown that the concepts of Fisher and Zahavi are not as mutually exclusive as most authors, Zahavi included, seem to assume. I find it hard to believe that whole groups like pheasants and birds of paradise have evolved and speciated successfully if their extraordinary sexual proceedings gain nothing for the participants *except* the plumes and wattles and super-long coiled tracheae, all of which seem so totally arbitrary, indeed deleterious for coping with the challenges of life in general. While not denying the potential for arbitrariness and exaggeration latent in gene-inspired choosing, utilitarian "good genes" as the original or ultimate objective of choice surely must exist.

Other small criticisms of the book concern lines of thought ignored to even worse degree than Zahavi's. But the first gripe is that it is hard to find out what has been overlooked because there is no author index. One's first use of such an index is, admittedly, almost always vanity; but even so author indexes are valuable, especially when the reference lists are separated at the end of each chapter as here. What I did still *not* find, even after thumbing the separate lists, was reference to the considerable statistical literature on stopping rules in sequential searches (e.g. *Am. Stat. Assoc.*

*J.*, March 1966). It was especially surprising to find no reference to this mathematical line of ideas even in Parker's original and useful chapter on mating decisions. Titles used in the literature — "The Dowry Problem", "The Beauty Contest" etc. — should alert mate-choice biologists, one would think. But perhaps a third title, "The Secretary Problem", hints at the reason for disinterest — too few biologists are in a position to be interviewing secretaries with a view to marrying the richest and prettiest.

My second and quite different criticism is that I could find no reference to disease contagion, venereal or otherwise, as a factor in mate choice. Freeland's (*Biotropica* 8, 12; 1976) discussion of this for primates is never mentioned, although a little charted sea may stretch out from here.

As corrective to these complaints, let it be said that many important topics *are* covered that are not mentioned in this review. Altogether the book is a well-made informative heap and every biologist at play in the meadow of its gathering, and others too, will want to climb on it. □

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## Balance of power

Ian Smart

**Energy: A Guidebook.**

By Janet Ramage.

Oxford University Press: 1983.

Pp.345. Hbk £15; pbk £5.95, \$12.95.

DR Ramage sets out to provide a comprehensive, balanced guide to all the most important features of the energy world, present and future, in terms intelligible to a reader with no technical background. She does not, of course, succeed. Within this extent, no one could. But she makes an absorbing effort. After briefly reviewing contemporary energy use, with 1980 as her focal year, she devotes more than half of her text to explaining the available technologies of energy conversion. A final section of the book then discusses the problems of foreseeing and planning for the future, while also allowing the author to tidy up some loose ends by importing a rather sketchy chapter on conservation and one or two additional supply and conversion options such as hydrogen and biogas.

Comprehensive, the book is not. Dr Ramage is most obviously enthusiastic, and splendidly lucid, when writing about the generation and use of electricity. Energy for transport, space heating and lighting receives rather less attention. And there is very little indeed about industrial process heat — which may help to account for the fact that the chapter on oil and

natural gas deals almost exclusively with motor vehicles. More seriously, the author's continual and often enlightening emphasis on efficiency is confined within the engineer's meaning of the term. Questions of economic cost and benefit are hardly mentioned, let alone addressed.

Intelligible, it is. Dr Ramage has been at great pains to explain the rudiments of both science and technology in simple terms. The lay reader may still have to work hard at times to apprehend a dense exposition, but the evidence is all here. Balanced, it also is — but at a price. Dr Ramage is scrupulous in setting out conflicting arguments, and extraordinarily discreet about her own conclusions. Inevitably, that will leave many of her readers dissatisfied. She tells them what to think about, but not what to think. Indeed, she deliberately obstructs any simple conviction about future policy, by insisting at every point on the complexity of the choices to be made and the uncertainty about their implications.

This is, in fact, a simple book that enormously complicates the subject it discusses. Such an inversion of the prevailing fashion is not the least of its attractions. And an author who writes "we shouldn't be fooled into believing that any number of pages of mathematics can produce reliable predictions from uncertain data and doubtful assumptions" (p.282) has at least one reader on her side. □

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## Tackling fuzziness and uncertainty

F.H. George

### Logic Machines and Diagrams.

By Martin Gardner.

*Harvester/University of Chicago Press: 1982. Pp.176. Hbk £15.95, \$15; pbk £4.95, \$5.95.*

MARTIN Gardner has written a very exciting book, one which anyone interested in what might broadly be called Information Technology should read. The emphasis is, as one might expect, primarily mathematical, taking us from the thirteenth-century world of Ramon Lull up to the present-day concepts of machine intelligence.

Mathematical logic plays a leading role in the book. Several chapters are devoted to methods of representing logic and logical arguments that are traditionally associated with the Euler-Venn diagrams (although Venn was associated primarily with a form of elliptical representation of class relationships more complicated than those circular representations associated primarily with Euler).

The part of the book of greatest current interest is to be found in a final chapter devoted to machine intelligence. Here such important matters as Robinson's Resolution method of theorem proving, and binary semantic trees are discussed, with probabilistic logic (now long established) and fuzzy logic (a much more recent development) also coming in for consideration. It is clear that the fuzziness in logic represents some of the fuzziness inherent in ordinary language; it serves as a reminder that this aspect of logic does not lie in the direction of the great traditional developments of mathematical logic, which were concerned primarily with the foundations of mathematics. It should be said that uncertainty in logical inference making can involve uncertainty over the class definition (e.g. the class of neurotic people) or over whether a particular member (person) belongs to that class (e.g. Is Charles neurotic?). The uncertainty over the latter point may merely occur because we do not know enough about Charles. Such uncertainty is represented in fuzzy logic.

Two especially thought-provoking questions are proposed by the author: "[is it] possible to formalize a computer language that will model fuzzy reasoning in useful ways"; and "is there a useful way . . . to diagram fuzzy logics with fuzzy diagrams?". The word "useful" stalks us in both these quotations, but certainly, as is acknowledged in the book, Zadeh himself (who first coined the expression "fuzzy system" in 1965) is working towards an answer to the first point. One can also think of a number of ways, using dotted lines and the like, that the second may be tackled,

although the usefulness of the results is uncertain. In both cases the key point seems to be, as with probabilities, that the language which describes uncertainty, which may or may not be measureable, is itself certain.

One assertion — perhaps the only one in the whole book — gave me pause, and cause for disagreement with Gardner: "How the human mind works remains, of course a profound mystery". Not really! We do not know with certainty, of course, but most people would say that we know a great deal about the mind, even if we are unsure of the exact workings of the mechanism.

This is a field where much remains to be done, though "profound mystery" is surely an exaggeration. But here, perhaps, Gardner has taken poetic licence. The question of Gödel's theorem and its relation to machine thinking has been disposed of and it is recognized that machines that hope to achieve human flexibility of thought must operate heuristically. Granted they can do so, there is no reason to argue that machines could not think and behave as intelligently as human beings can. All this is recognized by Martin Gardner, as is the close relationship between machine intelligence and philosophy, in this truly excellent book. □

*F.H. George is Head of the Department of Cybernetics at Brunel University.*

## Counting the cost

Joseph Rotblat

### The Medical Effects of Nuclear War.

The Report of the British Medical Association's Board of Science and Education.

*Wiley: 1983. Pp.188. Pbk £4.50, \$8.95.*

THE EFFECTS of nuclear war have been studied extensively for nearly four decades, and the argument has been advanced that we already know everything about the topic that there is to be known, at least in its scientific aspects. Indeed, at a recent debate in a prestigious scientific society in the UK this argument was successfully used to quash a project for further study.

It seems odd that such a view should be adopted by scientists in relation to a subject about which there is no experience, but which produces new phenomena at a rate of one per decade. Thus, it was not realized until the early 1960s that the malfunction of electronic equipment during nuclear tests was due to the electromagnetic pulse emitted at explosions. The depletion of the ozone layer in the stratosphere began to be discussed in the 1970s. And it was only a year ago that a new effect was predicted: the dramatic reduction of sunlight resulting from the injection into the troposphere of huge quantities of soot from the

## Colour Science in Television and Display Systems

W N Sproson

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numerous fires started by the explosions. This may yet prove to have the most detrimental effect on survival after a nuclear war. Surely these problems call for detailed, multi-disciplinary investigations on an international scale.

But even in the well-established effects — blast, heat and radiation — large gaps exist in our knowledge, allowing widely differing estimates of casualties to be made. For example, the Home Office calculates that in an attack on the UK with nearly 200 megatons, deaths from radiation exposure would number less than one million, whereas a group of independent scientists showed that about four million radiation fatalities might occur in the London area alone after a 13 megaton attack! Very considerable differences also appear in estimates of blast casualties.

The medical profession — nationally and internationally — is profoundly concerned about these issues, not least because doctors are being asked to participate in medical planning for the aftermaths of a nuclear war. In 1981 the Annual Representative Meeting of the British Medical Association passed a resolution calling for a review of the medical effects of nuclear war and the value of civil defence, to help the BMA in formulating a policy. A special Working Party studied the problem for 18 months; its results are now presented in *The Medical Effects of Nuclear War*.

In order to assess the magnitude of the medical problem, the Working Party first discussed the scale of a presumed Soviet attack on the UK. It concluded that the attack is likely to be of about 200 megatons, which corresponds to over 3 tons of high explosives for every inhabitant of these isles. This happens to be the same quantity as available in the nuclear arsenals for every inhabitant of the globe. Considering the large number of targets in the UK, an attack on this scale seems a very conservative estimate. Indeed, the Working Party adds that the level of attack might be several times higher should cruise missiles be deployed.

The next step was to calculate the short-term casualties from such an attack in the light of the divergent estimates mentioned above. After a detailed analysis, the conclusion reached was that the independent scientists — who used mainly semi-official American data — gave a more realistic estimate of the blast, heat and radiation effects than the Home Office. As a result of questioning of the Home Office, the latter is revising its calculations; this can be chalked up as an important concrete achievement of the BMA study.

The actual number of fatalities is estimated as between 25 and 40 million, depending on various assumptions made. From the point of view of medical involvement, the number of injured people (estimated to be several million) is of prime importance, but the exact number does not matter a great deal since even the most optimistic estimates far exceed the capability

of the medical profession to cope with the situation. Surprisingly, this aspect is not discussed as widely as might have been expected in a report from a medical group. For example, the difficulty of triage (segregation of victims into three groups: with a poor chance of survival; with a reasonable chance if treated; with a good chance even without treatment), which is bound to be a major problem, is mentioned but is not considered in detail.

In a revised edition of the book, these aspects ought to be given more weight (and greater care taken in the editing and proof-reading). But the conclusions are not likely to change: the National Health Service could not deal with the casualties even from a single one-megaton weapon. "It follows that multiple nuclear explosions over several, possibly many, cities would force a breakdown in medical services across the country as a whole". □

*Joseph Rotblat is Emeritus Professor of Physics at the University of London. He was Secretary-General of the Pugwash Conferences on Science and World Affairs from 1957 to 1973.*

## Tango with a quango

Philip Lowe

### **Policing Pollution: A Study of Regulation and Enforcement.**

By Genevra Richardson with Anthony Ogus and Paul Burrows.

Oxford University Press: 1983. Pp. 204. Hbk £15, \$34.95; pbk £6.95.

"MEMBERS of the public do not commonly concern themselves with the maintenance of sewage quality", remark the authors of *Policing Pollution*. Quite so. Instead, the necessary task of checking what is discharged into the public sewers of England and Wales is relegated to the Regional Water Authorities, set up in 1974. The sewers have to carry not only domestic sewage but industrial wastes, and that is where difficulties arise. For some liquid wastes may pose a health risk to sewerage personnel; other wastes may damage or overwhelm the capacity of treatment plants and thus pollute the watercourses which receive the treated effluent.

To prevent such occurrences, all discharges from trade premises must be licensed by the Water Authorities. The licences are called consents and they impose specific conditions on the composition, volume and rate of discharge of effluent. The Water Authorities are accorded considerable latitude in respect of the conditions and payments they may impose. They also have the power to prosecute any trader who discharges without consent or in breach of the consent conditions.

It is the way in which these wide discretionary powers are exercised that is the focus of *Policing Pollution*. One of the

authors, Genevra Richardson, spent several months with the enforcement officers of two Water Authorities interviewing them about their work. What emerges is the officers' extreme reluctance to use the legal sanctions available to them. Breaches of consent conditions are routinely overlooked. Indeed, one of the Water Authorities had never brought a prosecution, even though in some areas as many as 30% of all sampled effluents were technically criminal.

The reason was that the officers regarded co-operation, rather than confrontation, as the better means of achieving their objectives. Most breaches of consent conditions did not pose a real threat to the sewerage system or the people working in it, and therefore could be safely ignored. The officers were more concerned with what might happen if their relationship with local traders broke down. The ability of industry to "pull out the plug" seemed a constant threat to be averted only by careful negotiation and mutual understanding. "If we lose co-operation, we lose control", one senior officer explained.

Law enforcement was regarded as a means of preventing harm, rather than an end in itself. This attitude fitted in with the view that officers had of themselves as technical experts helping industry overcome its treatment problems. As one officer put it: "We come in as consultants rather than as stick-wavers". They were certainly very sensitive to the practical problems, both technical and financial, faced by firms making an effort to comply. Prosecution was regarded as a mark of failure — a last resort to be used only against rogue firms.

The book breaks new ground in the empirical analysis of the enforcement of regulatory controls, demonstrating the subordinate role of the criminal law and the extensive use of non-criminal sanctions. However, it has shortcomings. No survey of traders' attitudes to enforcement was carried out, even though perceptions of these attitudes were so salient in determining the attitudes of the enforcement officers. The effectiveness of enforcement is not evaluated, despite the opening chapter's general exposition of the economics of government regulation and the claims made in the third chapter for the superiority of Britain's flexible and non-legalistic approach to pollution control.

Finally, there is no effort to draw on social or political theory to characterize the relationship between the enforcement authority and industry. It is a relationship which resembles a couple performing the tango: first one takes the lead, the other responds; and then vice versa. But throughout they remain locked in a firm embrace. □

*Philip Lowe is Lecturer in Countryside Planning at University College London. His book Environmental Groups in Politics, co-written with Jane Goyder, was published earlier this year by George Allen & Unwin.*



# Local area networks

*Local area networks are increasingly being used to link data-processing equipment.*

*Adrian Stokes reviews their history and their prospects*

WITH the increasing tendency for computers, terminals and laboratory recorders to be developed which have to inter-communicate, there has grown a need for providing a more sophisticated means of communication between them. In the past, it has often been enough to connect a single item of laboratory equipment through a four-wire cable into a dedicated minicomputer. Now, however, a large number of different devices, often with relatively incompatible interfaces, may have to inter-connect, rendering the single cable obsolete. To meet the need for sophisticated inter-linking, a system of "local area networks" has been devised.

## Wide area networks

Twenty years ago, computers functioned independently of one another and the only form of "network" was a series of terminals which could access that computer, sometimes over long distances, by means of the public switched telephone network.

In 1966, Marill and Roberts<sup>1</sup> published the results of their experiments in connecting two computers — the TX-2 computer at the Lincoln Laboratory in Lexington, Massachusetts, USA and the Q-32 machine at System Development Corporation, Santa Monica, California, USA. But these experiments, although interesting, were of little practical use, partly because, according to Roberts<sup>2</sup>: "... The degradation in response caused by using telegraph or voice-grade communication lines for network connections is significant enough to discourage most users, ... (and partly because) the cost to fully inter-connect computers nationwide either with direct-leased lines or dial-up facilities is prohibitive."

Nevertheless, the Advanced Research Projects Agency (ARPA) of the US defence department set up an experimental network which was the precursor of a great many "wide area networks". Now, most developed countries have at least one wide area network.

Wide area networks are characterized by the geographic spread of the network, which is large, and by the rate of data transmission, or "bandwidth", which is relatively small (often of the order of 50 kbit per second). A complementary technology is that of "local area networks" which cover a restricted geographic area but which are able to transmit data at tens of megabits per second.

## Local area networks

There are several different types of local area networks (Fig.1). Two broad categories are of some importance. The first uses a centralized switch, and is based on the ubiquitous telephone exchange. The second uses a distributed approach and is the type most often considered when the term "local area network" is used.

Modern telephone exchanges use computer technology heavily and many use digital switching within the exchange. Transmission to the exchange is either analog (in which case, standard telephones can be used) or digital (in which case, the telephone instrument contains a simple analog-to-digital converter — a "codec"). In the former case, it is clearly possible to transmit data (by use of a modem) but the data rates possible are fairly low. If digital transmission is employed, a bandwidth of 64 kbit per second is required, which means that this bandwidth is also available for data transmission.

A variation on the telephone exchange, or PABX, is the private automatic computer exchange, or PACX, which is intended solely for data transmission. In this case, the connection is made by means of control signals within the data.

Use of such technology has many advantages: in particular, the PABX allows data and voice to share the same exchange. Also, the system can use wiring which is already in existence. However, a clear disadvantage of the PABXs in many applications is that they use "circuit-switching" whereby the sender of data must initiate a "call" to the recipient, and this is then a

point-to-point connection.

More usually, the term "local area network" is applied to a more restricted type of technology, exemplified by the Cambridge Ring<sup>3</sup> and the Ethernet<sup>4</sup>. These use a dedicated transmission medium and permit the transmission of data at high bandwidths (of the order of 10 Mbit per second). They use "packet-switching" techniques, whereby the sender breaks down the data into packets and then indicates their destination. It is therefore possible to send data to a number of different destinations using a single connection. In addition, the system does not rely on a single component such as the exchange itself. Indeed, in the case of Ethernet, the transmission medium (a co-axial cable) is passive and so new items of equipment can be added to or taken away from the network without interrupting its operation.

The Cambridge Ring, at its simplest, consists of a twisted pair-cable (in fact, optic fibres are often used) along which data is transmitted from one "ring station" to the next. At any one time, the ring has a number of circulating packets of data which may be empty or full. When a ring station wishes to transmit data, it places the information into an empty packet, and then marks it as filled. The packet contains a destination address and, when the packet reaches this address, it is marked as "received", but not as "empty". It then returns to the sender, which notes that it has been received, and then marks it as "empty". Ethernet, on the other hand, is a broadcast system. It uses a co-axial cable as its transmission medium and its topology is that of a tree without roots. The specific

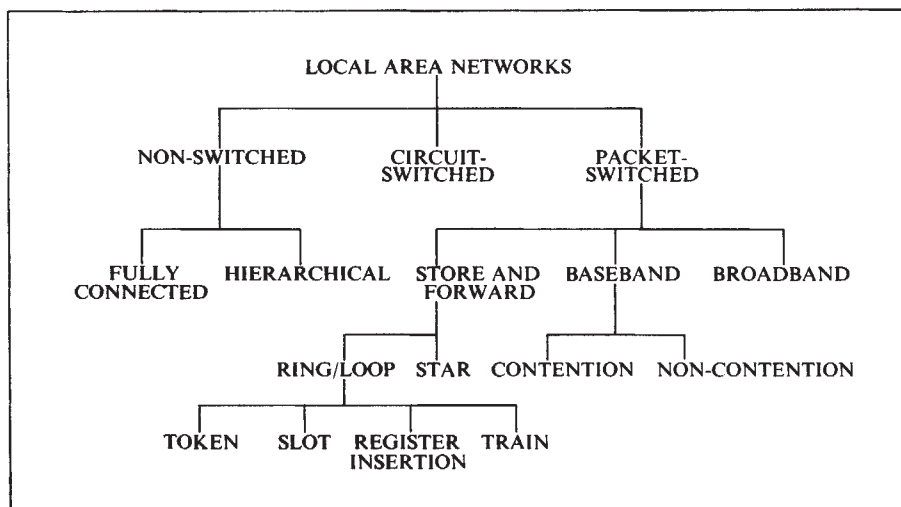


Fig.1 Outline of the types of local area networks

constraint of this system is that there must be a single path between any source and any destination. Data packets are broadcast onto the "ether" and discarded by all stations except the one addressed in the packet. Should two packets be transmitted at the same time, they will become corrupted, and will therefore be discarded by all stations and retransmitted later. The most common protocol employed on Ethernets is CSMA/CD (carrier sense multiple access with collision detect).

Local area networks are available commercially, and the two different types of technology are currently being evaluated in the British National Health Service — the Ethernet at St Thomas' Hospital<sup>5</sup> and the Cambridge Ring at the Royal Postgraduate Medical School, both in London.

### Future applications

A major disadvantage of this technology at present is its cost, but this should drop as special-purpose "chips" become available. However, a number of other products are coming onto the market which have many of the features of local area networks but which are considerably cheaper. One of these is the product CP/Net which allows the interconnection and networking of all microcomputers which run the standard CP/M operating system.

As computers become more widely used, there is a growing need to provide remote access to them, and so it is likely that a hierarchy of networks will evolve in the next few years. A terminal on a desk (or a piece of laboratory equipment) will be linked into a low-bandwidth network within the immediate room or building which will be connected into a local area network of high bandwidth covering the entire site. This will be connected into the national PTT network which will itself be connected to international data networks. Such a hierarchy has a clear analogy with the telephone system.

Thus, in a relatively short time, there is likely to be an "information" plug in most laboratories and offices in much the same way as there are now plugs for electricity. Various items of equipment (with standard interfaces) could be connected to such a plug, and could access or transmit data worldwide.

*Dr Adrian Stokes is director of computing at St Thomas' Hospital, London, UK, and King's Fund Fellow.*

## Computers and data processors

- The model 705 scanner from Keithley Instruments is designed for use with the IEEE 488 interface. The 705 scanner can scan up to 40 channels and allows for 1, 2 and 4 pole operation. Its "daisy chain" design allows more scanner units to be added without requiring additional IEEE address; units can be designated as either master or slave. A variety of plug-in scanner cards are available, allowing measurement from low voltage (200 nV), low current (1pA), general purpose and thermocouple inputs. The equipment has three scanning modes, external triggers and digital input/output ports.

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- LeCroy Research Systems has developed a processor which will solve data rate problems either by trigger decisions or by compacting the data before it is read out by an on-line computer. This 4800 series CAB also allows systems to be created using parallel or tree architectures with data crunching potential at each node. It has a bit slice processor that provides multi-operation and multi-operand instructions with a 200 ns basic cycle time, including 16 × 16-bit multiply and 16-bit shift operations. The CAB system includes a 4 kbit × 24 bit RAM data memory and a 4 kbit × 24 bit RAM instruction memory.

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## Integrators and converters

- A new computing integrator, the CI-10, has been released by LDC/Milton Roy. It has a 10 ms slicing speed for use in capillary gas chromatography and HPLC. Start-up is made easy through a set of built-in default parameters and a 16-character alphanumeric display. Up to 9 methods and over 800 peaks can be stored in the 32 kbit memory.

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- The new 4270 integrator from Varian Associates is designed for data handling in routine chromatographic assays. The instrument can be used as a single-channel computing integrator or, with an optional module, as a dual-channel integrator. It has an 8½ inch thermal printer/plotter, 16 kbyte of RAM and 48 kbyte of ROM for system software, and 50 or 60 Hz data sampling.

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- A new 8-bit video digital-to-analog converter has been announced by Analogic. The model AH8308E is designed for colour or monochromatic digital video systems. It has an improved temperature stability and reduced drift. It also has an update rate of 150 MHz.

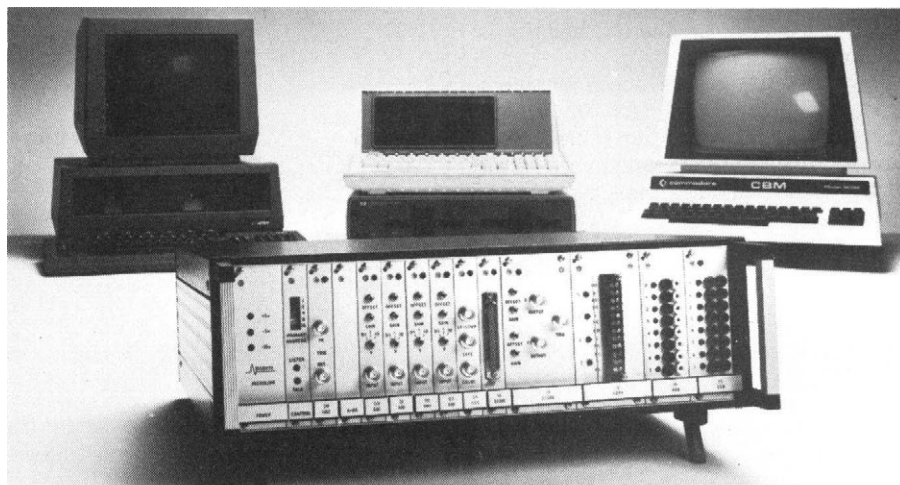
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- The latest addition to the Linseis range is a data acquisition system which can take up analog signals and transmit them directly to a computer. It is available in a version taking between one and six signals, and consists of a basic housing plus various plug-in modules. All signals to be measured are taken up by the input modules and standardized to a level of 0–1 V; they are then converted into digital signals and passed on to the computer via a V24 interface (RS 232) or an IEC bus. To convert the instrument to accept different signals, a new input module is needed. These modules are interchangeable and are available for almost every type of measuring signal. Variations on the modules may also be supplied.

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- Biodata's Microlink is a computer interface specifically designed for use in a laboratory environment. It is fully modular, consisting of a mainframe containing an IEEE 488 interface and cabinet into which up to 18 modules can be plugged. Several modules are also available, including thermocouple inputs, root mean square inputs, time counters, 12-bit converters and RS 232 converters.

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*The Microlink computer interface from Biodata*

1. Marill, T. & Roberts, L.G. *Proc. AFIPS FJCC* 29, 425–431 (1966).
2. Roberts, L.G. & Wessler, B.D. *Proc. AFIPS SJCC* 36, 543–549 (1970).
3. Wilkes, M.V. & Wheeler, D.J. *Proc. Local Area Networks Symp.*, Boston, 47–62 (1979).
4. Metcalfe, R.M. & Boggs, D.R. *Ethernet: Distributed Packet Switching for Local Computer Networks*, *Comm. ACM* 19(7), 395–404 (1976).
5. Stokes, A.V., *St Thomas' Hospital Local Area Network, Communicate*, 19–21 (1983).



## Accessories

● Two new products have been released by Le Croy — a microamplifier and a multi-source time interval meter. The model TRA403 microamplifier provides a gain of 330 mV per amp with a 7 ns response time, and dissipates around 25 mW per channel. Noise levels are kept to 25 nA (root mean square). The CAMAC model 4204 multi-source time interval meter includes a 16-channel time-to-digital converter that accepts up to 16 different sources of stop or start for one time interval measurement.

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● Cambridge Instruments have produced two new systems, designed for very large scale integration (VLSI) and computer aided design (CAD), named Chiprite and Chipcheck. Chiprite is an electron beam lithography system that writes reticles, masks and wafers using information directly from CAD tapes. Chipcheck is an automatic inspection system that compares the information on the CAD tapes with the mask or reticle and automatically identifies and classifies any defects.

*Circle No. 108 on Reader Service Card*

● The model 4416 multi-instrument data system is a new chromatography analyser from Nelson Analytical. It is based on the 16-bit HP 200 series model 16 desktop computer and includes a VDU, dual micro-floppy disk drive, a printer/plotter, chromatography software and an instrument interface.

*Circle No. 109 on Reader Service Card*

● As part of their computer assisted measurement and control (CAMAC) package, Transiac have introduced a complementary pair of modules. The first, the microprocessor-controlled digital voltmeter (DVM) model 2032 uses a simultaneous ditherizing/digital filtering technique which increases the accuracy of the 12-bit system to 14 bits, and suppresses the power supply noise by 100 dB. The on-board processor also provides for auto-ranging in each channel. It is also self-calibrating with the answer specified in volts. The second, the model 3016, feeds back process control signals from the DVM to the system with a 16-bit "settability", and a guaranteed 14-bit performance.

*Circle No. 110 on Reader Service Card*

● The MPNK-72-1 prom-programmable word processor keyboard has been introduced by Amkey. This is a 72-key solid-state capacitance keyboard featuring an 8048 or an 8748 microprocessor, of which the 8748 is code-programmable. The MPNK-72-1 is available with any one of five different connectors, including a card edge, a four- or six-pin modular telephone jack or a four- or six-pin 90° header.

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*Digital Engineering's new bit-map graphics products*

● A new line of bit-map graphics products has been announced by Digital Engineering. Designated the 512-series Retro-Graphics, the printed circuit board kits give Tektronix 4010/4014 graphics-terminal emulation and are compatible with Tek Plot 10 graphics software on TeleVideo, Adds, and Lear Siegler terminals. The 512-series is compatible with RG512-based programs. The 512-series provides incremental point plotting, multi-style vector drawing (dotted, dashed, and solid), four graphics character sets, selective erase, crosshair cursor, and optional light pen functions. The new Digital Engineering models are compatible with most popular applications and utility graphics software programs, including DISSPLA, TELLAGRAF, Plot 10, Template, D1-3000, SAS/GRAF, and ILS.

*Circle No. 112 on Reader Service Card*

● Altek has added 48×80 and 42×130 inch digitizers to their standard line of Datatab tables. The digitizers have an accuracy of ±0.05 inches within any 48 inches, and resolution and repeatability of .001 inch. Altek's large-sized Datatab digitizers can be used with any of the Altek controllers.

*Circle No. 113 on Reader Service Card*

● A system that protects software for the new Commodore 8000 SK series computer has been developed by Mektronic Consultants. This new Safeware system is a combined hardware and software package which consists of a user port plug-on unit and an interrogation routine which can be slotted in anywhere in the program and will only run if the hardware unit is plugged in.

*Circle No. 114 on Reader Service Card*

● A 256 kbit dynamic random access memory (RAM) card has been introduced by Zenith Data Systems for its Z-100 series of desktop computers. The new card is IEEE 696 compatible and therefore also able to run in many S-100 bus computers such as the CompuPro, Dynabyte, Northstar or Vector Graphics computers.

*Circle No. 115 on Reader Service Card*

● A low-profile 8-inch flexible disk drive is now available from Zenith Data Systems in both single- or dual-drive versions. The new drives are double-sided, double-density and offer 1 Mbyte of storage per diskette. Single drive versions can be upgraded to add a second drive when desired. The Zenith drives are half-height Shugart SA860 disk drives and can be used with any desktop computer having a standard 8-inch drive controller.

*Circle No. 116 on Reader Service Card*

● The Ancom series 5000 monitoring, datalogging and control Eurocard system has now been expanded to cover the measurement and control of temperature, voltage, humidity and pressure and other control system parameters. Based on the single Eurocard standard, variations from a single input measuring to a 1,000 channel multi-function, Z80-controlled system, can be assembled. Inputs are multiplexed using the Ancom multiplexer bus. An isothermal box is included at the front of the card for thermocouple connection and there is auto-cold junction compensation. Other examples of input cards in the 5000 series include the RTD input series for 2, 3 and 4 wire platinum RTDs, with typical accuracy figures of ± 0.1 °C, and both electronic and wet-bulb relative humidity sensor conditioners. The system can be interfaced for communication to other computers and systems and to mass storage media, such as an external paper tape punch or disk drive. Processing options now include analog-to-digital and voltage-to-frequency cards, and there is a range of amplifier, filter, microprocessor and memory options available. The 5000 hardware series is supplied with a software package to control and program the system.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.



## Software

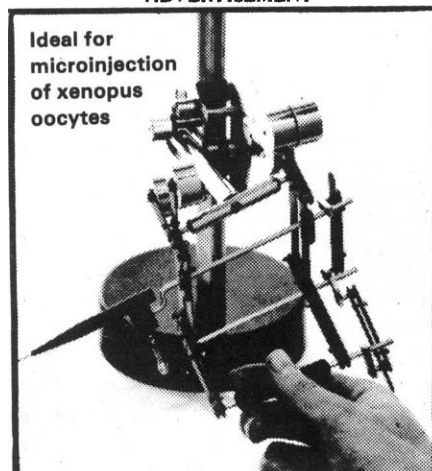
● Zenith Data Systems are now marketing the Lotus 1-2-3 integrated software package for use on the Z-100 family of desktop computers. Used with the Z-100, the Lotus 1-2-3 gives an eight-colour presentation. Lotus files on compatible disk formats, such as that of the IBM personal computer (PC), can be read and manipulated directly on the Z-100. The Lotus 1-2-3 can also be used on Zenith's new Z-100 models with built-in Winchester drive, which have a storage capacity of 10 Mbyte and 192 kbit of RAM. The 1-2-3 includes a spreadsheet, data base and graphics capabilities.

*Circle No. 118 on Reader Service Card*

● A new chromatography software package based on the IBM personal computer (PC) has been introduced by Nelson Analytical. The IBM PC chromatography software package includes programs for establishing methods, data acquisition parameters and calculation routines. The software allows complete storage of raw data and can work out area percentages, internal and external standard and normalized area percentages along with giving a graphic display of the chromatograms with baselines. Post-run routines allow recall and comparison of stored runs, subtraction, ratios and altering baselines with recalculations. The software package is for use with a Nelson Analytical model 861 or 862 intelligent interface — an analog-to-digital interface with 20-bit precision and memory for buffering chromatography.

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● New revision software has been produced by UCL (Universal Computers Limited) which updates their Ultimate computer. The new features included in the Revision 110 software are a type-ahead option, which means that the computer can store instructions of up to 224 characters in addition to the instructions on which it is working; a read-status verb, which allows the operator to look at the status of each line on the system; the drop-DTR and raise-DTR verbs which can, for example, fix the status line if it has hung; the drop-RTS and raise-RTS verbs which can restore a line to normal running status; a "spooler" which can handle up to 20 printers simultaneously; multiple tape drives and a T-copy verb which allows the user to make tape copies at the maximum speed of the tape drive.

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● A new software package that reduces the cost of preparing and monitoring Atlas automatic test programs has recently been introduced by Thorn EMI Electronics. Designated Toolchest, this new package lowers the level of Atlas knowledge required of writers. The Toolchest is aligned to the latest international IEEE standard — IEEE 416-1981 (level 16).

*Circle No. 121 on Reader Service Card*

● Microcomputer software for predicting outdoor noise levels has been developed by Battelle's Columbus Laboratories. The programs calculate atmospheric attenuation from specified meteorological conditions. Information from up to 15 noise sources and receivers can be analysed to determine both overall noise levels and the relative contribution of each source.

*Circle No. 122 on Reader Service Card*

## Accessories for Apple microcomputers

● Cyborg Corporation has announced that its new model 41A Isaac is compatible with the Apple IIe personal computer, as well as with the Apple II. The model 41A Isaac has an extended BASIC software package, a new high-speed data acquisition facility and a graph formatting program. The new model 41A is modular, so that only certain capabilities need be purchased at one time, and the system expanded later.

*Circle No. 123 on Reader Service Card*

● A chromatography data system is now available from Heyden Datasystems. Chromcard, an integrated hardware and software system, connects Apple II series computers to any chromatograph. It acquires data and then produces a laboratory report. The Chromcard will connect to any gas or liquid chromatograph. It will plot chromatograms on the Apple screen; provide tick marks on the recorded output to show peak integration start and finish; it reports peak height, peak area and retention time for each component. In addition it can be used to perform internal standard calculations, print full analytical reports and store report and data handling methods on disk.

*Circle No. 124 on Reader Service Card*

● A new Apple II series software package called 'Quick Search Librarian (QSL)', has been announced by Heyden Datasystems. The main features of QSL include a typical search speed of 50 articles per second; a typical sorting speed of 40 articles per second, and the storage of 1,000 articles and descriptions on each disk.

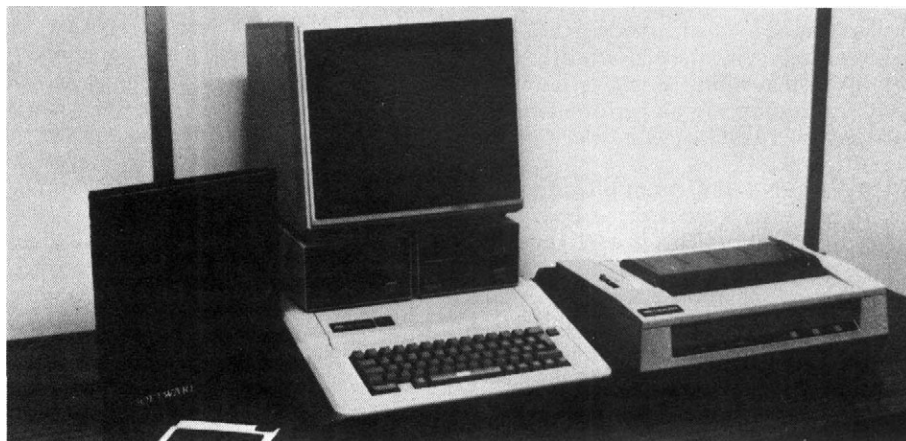
*Circle No. 125 on Reader Service Card*

● Amkey has produced the PRO-100 detachable capacitance-keyboard with enclosure for use with the Apple II and II+ personal computers. The PRO-100 offers 100 keys supporting all existing Apple functions as well as a horizontal and vertical cursor movement, a separate number pad with entry key, an auto-repeat, a relocated reset key, a CAP lock key, a power-on indicator and 22 Visicalc keys.

*Circle No. 126 on Reader Service Card*

● API have developed a new version of their Apilab computer program which will run on Apple microcomputers. The Apilab program was originally developed to run on Commodore Pet computers and it contains the databases for three products — Api 20E, Rapid 20E and Api 20Strep.

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*API's new Apilab computer program*